

Summary Report

AFNOR validation of the Genesystems method for the detection and quantification of *Legionella pneumophila* in waters

- CAE Central Laboratory

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 pneumophila* in waters

1. PURPOSE OF THE STUDY

The present study comply to AFNOR's validation of the GeneSystems *Legionella pneumophila* method for the detection and quantification of *Legionella pneumophila* by PCR in waters. It has been carried out in accordance with the « validation protocol for detection kits and counting of *Legionella* et *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. Extraction recovery, PCR step detection and quantification limits, quantification linearity, detection and quantification inclusivity and exclusivity, as well as feasibility were examined in particular. Phase 3 includes a ring trial study to assess the results obtained and the fidelity (repeatability and reproducibility) of the supplier's method and protocol by statistical analysis.

1.1. STANDARD OF VALIDATION

The AFNOR validation protocol is based on the criteria, experimental plans 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 polymerisation 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 step of DNA quantification of *Legionella pneumophila* or *Legionella* spp by real time PCR with the GeneDisc Cycler 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 Cycler.

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 Cycler® 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 Cycler 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 Cycler software automatically calculates the Ct, standard curve parameters and the second degree polynomial representing the: Inhibition controls fluorescence amplitude curve = $f(\text{Ct } L. \text{ pneumophila})$. 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 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.

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

The GeneExtract[®] and the GeneDisc Cyclor[®] 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
Day 1	8.02	0.138	7.96	0.084	8.28
Day 2	8.80	0.104	8.40	0.161	8.81
Day 3	8.54	0.473	8.57	0.299	8.96

* 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
Evian water	1 000	117	75		
	10 000	45			
	100 000	63			
ECS	1 000	102	64		0.28
	10 000	58			
	100 000	32			
TAR	1 000	84	66		
	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 of a concentration estimated at 5 GU/well were tested. Amplification and detection were carried out on the consumable intended for the detection of *Legionella pneumophila* (*Legionella pneumophila* Premium GeneDisc pack – Ref. GDLP-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 of a concentration estimated at 25 GU/well was analysed 30 times, within repeatability requirements. Amplification and detection were carried out on the « *Legionella pneumophila* GeneDisc *Premium* pack» kit.

- Quantification limit of *L. pneumophila* GeneDisc *Premium*

	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*
Validation criteria					< 0.50	<2.045	<0.30
Results obtained	25	1.39	23	0.03	0.288	2.338	0.15
Conclusion					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 ranges were achieved from 5 standard genomic DNA of *L. pneumophila* ATCC33152, supplied by Genesystems (SDNA-Lp). A linearity study was achieved for values 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 / 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 regression	2509.6	4.35	compliant
F model error	0.24	3.10	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	
Average bias	0.016	-0.034	0.019	-0.001	-0.037
standard deviation	0.040	0.086	0.061	0.019	0.067
Elin*	+/- 0.04 Log	+/- 0.09 Log	+/- 0.06 Log	+/- 0.02 Log	+/- 0.08 Log
Uncertainty	+/-0.08	+/-0.18	+/-0.12	+/-0.04	+/-0.16

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

CONCLUSION

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

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*.

Bacterial strain	Genedisc Premium pack <i>Legionella pneumophila</i>
<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 maltophila</i>	Absence
<i>Xanthomonas</i>	Absence

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

Bacterial strain	Genedisc Premium pack <i>Legionella pneumophila</i>
<i>L.anisa</i>	Absence
<i>L.birminghamsis</i>	Absence
<i>L.bozemanii1</i>	Absence
<i>L.bozemanii 2</i>	Absence
<i>L.cherrii</i>	Absence
<i>L.cincinnatiensis</i>	Absence
<i>L.dumofii</i>	Absence
<i>L.erythra 2</i>	Absence
<i>L.feeleii1-2</i>	Absence

<i>L.gormanii</i>	Absence
<i>L.hackeliae</i> 1-2	Absence
<i>L.jordanis</i>	Absence
<i>L.lansingensis</i>	Absence
<i>L.longbeachae</i> 1-2	Absence
<i>L.maceachernii</i>	Absence
<i>L.micdadei</i>	Absence
<i>L.oackridgensis</i>	Absence
<i>L.pariensis</i>	Absence
<i>L.sainthelensis</i> 1-2	Absence
<i>L.tucsonensis</i>	Absence
<i>L.wadsworthii</i>	Absence
<i>L.pneumophila</i> s1	Presence
<i>L.pneumophila</i> s2	Presence
<i>L.pneumophila</i> s3	Presence
<i>L.pneumophila</i> s4	Presence
<i>L.pneumophila</i> s5	Presence
<i>L.pneumophila</i> s6	Presence
<i>L.pneumophila</i> s7	Presence
<i>L.pneumophila</i> s8	Presence
<i>L.pneumophila</i> s9	Presence
<i>L.pneumophila</i> s10	Presence
<i>L.pneumophila</i> s11	Presence
<i>L.pneumophila</i> s12	Presence
<i>L.pneumophila</i> s13	Presence
<i>L.pneumophila</i> s14	Presence
<i>L.pneumophila</i> s15	Presence

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.

- **Premium Genedisc 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 Cycloer[®] 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 and in the event of inhibition, it is 10µl.

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. It is a matter of calibrated oligonucleotides including specific primers from *Legionella pneumophila*.

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

The aim of this study is to assess the fidelity (repeatability and reproducibility) of kits supplied by Genesystems for the detection of *Legionella pneumophila*. All laboratories participating in this study use Genesystems technology.

3.2. TEST PLAN AND ANALYSES PERFORMED

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).

Detection of *Legionella pneumophila* was carried out for the three phases.

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 below summarises the main information for the *L.pneumophila* parameter.

Valeurs de fidélité - *Legionella pneumophila* :

Parameter	<i>Legionella pneumophila</i>	<i>Legionella pneumophila</i>	<i>Legionella pneumophila</i>	<i>Legionella pneumophila</i>	<i>Legionella pneumophila</i>
Sample	A	B	C	D	E
Number of laboratories retained	14	14	15	14	11
Initial	16	16	16	16	16
M = General average (n/l)	176 420	1 854 464	55 968	550 357	116 858
Sr (n/l)	25 047	226 966	16 264	146 341	34 276
SR (n/l)	52 490	491 431	53 013	574 398	102 609
CVr (%)	14,2%	12,2%	29,1%	26,6%	29,3%
CVR (%)	29,8%	26,5%	94,7%	104,4%	87,8%
M = General average (in log)	5,228	6,255	4,577	5,512	4,908
Sr (in log)	0,068	0,056	0,104	0,120	0,159
SR (in log)	0,134	0,110	0,414	0,493	0,414
CVr (%)	1,3%	0,9%	2,3%	2,2%	3,2%
CVR (%)	2,6%	1,8%	9,0%	8,9%	8,4%

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 came to light following 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.

The standard deviations of repeatability demonstrate that the entire method is repeatable since the maximal value obtained is 0.159 Log (sample E).

Finally, the reproducibility standard deviations express the samples' degree of complexity. Indeed, the maximal standard deviation obtained for the analysis of DNA solutions is 0.134 Log and it can reach 0.493 for samples taking all analysis steps into account (DNA preparations and amplification).

CONCLUSION

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

3. CONCLUSION

Results from the preliminary study, completed by the Laboratory Specialist, as well as the ring trial show that performances from the Genesystems method for the detection of *Legionella pneumophila* comply with the requirements of the XP T 90-471 standard.

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

PART 2: AFNOR extended validation of the Genesystems method for the detection and quantification of *Legionella pneumophila* in waters

1. PURPOSE OF THE STUDY

The present study looks at extending the AFNOR validation of the GeneSystems *Legionella pneumophila* method for the detection and quantification of *Legionella pneumophila* 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 plans 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 by polymerization chain reaction (PCR).

1.2. GENESYSTEMS METHOD PRINCIPLE

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

- Extraction of bacterial DNA:
 - 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.

The DNA from *Legionella* is then quantified by Real time PCR with the assistance of *Legionella* GeneDiscs Packs.

The GeneSystems *Legionella* PCR test is based on genetic amplification by real time PCR, of a specific nucleic sequence of the *Legionella* spp or the *L. pneumophila* classifications. Detection is possible owing to the use of TaqMan[®] 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 out fluorescence. This fluorescence is measured directly by the GeneDisc Cycleur optic module.

The DNA/Master Mix mixture, deposited in a GeneDisc sector, is depressed in 6 reactional chambers, pre-loaded in reagents (primers, probes, DNA) for each analysis. The composition of the 6 reactional chambers for 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 Cyclor 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

Recovery was assessed in order to verify the method's compliance. It was assessed for 6 independent samples, three different matrices and three different concentration levels. 54 samples were analysed in total. Recovery was evaluated 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
Day 1	8.39	0.17	7.7
Day 2	8.34	0.03	7.8
Day 3	8.55	0.19	7.9

* 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 gap-type (S _R)	Uncertainty ⁽¹⁾
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				

⁽¹⁾ 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

The detection limit was tested for the “Legionella pneumophila GeneDisc pack” (Ref. GDLPN-471-12 HT L. pneumophila) intended for the detection of *L.pneumophila* (Ref. GDLPN-471-12 HT L. pneumophila).

As regards the 30 tests achieved in duplicate, there was no negative result.

CONCLUSION

The detection limit is validated at 5 GU/well for the “Legionella pneumophila GeneDisc pack” (Ref. GDLPN-471-12 HT L. pneumophila), in compliance with the performances notified by the supplier.

2.3. QUANTIFICATION LIMIT

In the same way as for the detection limit, the quantification limit of 25 GU/well was tested with the “Legionella pneumophila GeneDisc pack” (Ref. GDLPN-471-12 HT L. pneumophila) intended for the detection of *L.pneumophila*. Thirty in duplicate analyses were achieved in accordance with repeatability requirements.

- Results obtained with the “*L. pneumophila* GeneDisc Pack”

	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*
Validation criteria					< 0.50	< 2.045	< 0.30
Results obtained	25	1.39	23	0.03	0.390	0.118	0.208
Conclusion					compliant	compliant	compliant

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

CONCLUSION

When the PCR analysis is achieved in two wells, the quantification limit is repeatable and accurate at 25 GU/well for the two GeneDisc Packs (*Legionella spp.* et *L. pneumophila*). In terms of measurement uncertainty, it complies with the new statistical model in the standard XP T90-471 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
-3.392	-4.115 < a < -2.839 75% < E < 125%	38.773	Compliant
Statistical analysis of linear model			
Origin	Value observed	Critical value With $\alpha = 5\%$	Conclusion
F regression	10444.6	4.35	Non compliant
F model error	4.13	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	0.060	-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
Uncertainty	+/- 0.18 Log	+/- 0.09 Log	+/- 0.25 Log	+/- 0.07 Log	+/- 0.15 Log

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

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 were tested for the “Legionella pneumophila GeneDisc pack” (Ref. GDLPN-471-12 HT *L. pneumophila*).

- Initial bacterial strains not belonging to the *Legionella pneumophila* classification

Bacterial strain	<i>L.pneumophila</i> GeneDisc Pack
<i>Aeromonas hydrophila</i>	Absence
<i>E.coli</i>	Absence
<i>P.aeruginosa</i>	Absence
<i>Alcaligenes faecalis</i>	Absence
<i>Flavobacterium</i>	Absence
<i>P.fluorescens</i>	Absence
<i>Bacillus subtilis</i>	Absence
<i>Klebsiella oxytoca</i>	Absence
<i>Pseudomonas putida</i>	Absence
<i>Burkholderia cepacia</i>	Absence
<i>L.monocytogenes</i>	Absence
<i>Serratia marcescens</i>	Absence
<i>Clostridium</i>	Absence
<i>S.maltophilia</i>	Absence
<i>E.aerogenes</i>	Absence

Proteus vulgaris	Absence
Xanthomonas	Absence
L.micdadei	Absence
L.bozemanii	Absence
L.jordanis	Absence
L.dunmofii	Absence
L.gormanii	Absence
L.parisiensis	Absence
L.anisa	Absence
L.longbeachae	Absence
L.tucsonensis	Absence

- Bacterial strains belonging to the *Legionella pneumophila* species

Bacterial strain	<i>Legionella pneumophila</i>
<i>L.pneumophila</i> s1	Presence
<i>L.pneumophila</i> s2	Presence
<i>L.pneumophila</i> s3	Presence
<i>L.pneumophila</i> s4	Presence
<i>L.pneumophila</i> s5	Presence
<i>L.pneumophila</i> s6	Presence
<i>L.pneumophila</i> s7	Presence
<i>L.pneumophila</i> s8	Presence
<i>L.pneumophila</i> s9	Presence
<i>L.pneumophila</i> s10	Presence
<i>L.pneumophila</i> s11	Presence
<i>L.pneumophila</i> s12	Presence
<i>L.pneumophila</i> s13	Presence
<i>L.pneumophila</i> s14	Presence
<i>L.pneumophila</i> s15	Presence
Modified Bacterial strains 1*	Presence
Modified Bacterial strains 2*	Presence
Modified Bacterial strains 3*	Presence

*: strains of mutant *L.pneumophila* not detected on the old GeneDisc version.

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**

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

There are two GeneDiscs 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 PRODUCTS SHELF LIFE.

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

GeneDiscs 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

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

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

REAGENTS READY FOR USE OR TO BE RE-FORMED

✓ **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 cuve must be validated in advance 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 $-20^{\circ}\text{C} \pm 3^{\circ}\text{C}$ 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

The results are preserved in computer files and/or on paper. Steps other than PCR are traced in documents expected at the laboratory. GeneDisc Cyclo[®] 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.

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 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

Results from the Expert Laboratory demonstrate that performances comply with XP T 90-471 standard requirements concerning the assessment of the Genesystems *Legionella pneumophila* detection method. At the end of this validation, the Genesystems method received certification no GEN 25/03-12/07 from AFNOR Validation. The GeneSystems *Legionella* pneumophila method is applicable for any type of water, without restrictions on use.