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Validation of analysis methods

Application to water microbiology

Validation protocol for commercial methods of detection and quantification of *Legionella* and *Legionella pneumophila* by concentration and gene amplification by polymerase chain reaction (PCR)

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(after approval by the relevant Technical Board)

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Scope

This validation protocol allows a method to be validated according to the requirements of Standards NF T90-471 and ISO/TS 12869. It applies to the detection and/or quantification of *Legionella* in water.

The methods to which this document applies are **commercial methods** (also called "**kits**" in Standard NF T90-471).

The purpose of certification is to demonstrate that the performance of these methods meets standard requirements.

The validation of a commercial method simultaneously concerns the operating procedure prescribed by the supplier, the test substances and equipment needed to implement the method, and a stated domain of application.

Principle

The validation study is based on criteria, experimental plans and calculation methods set by Standards NF T90-471 and ISO/TS 12869. The corresponding section references are given below, with some adjustments and additions.

The study comprises three phases, which are described in the document that follows. According to the composition of the method to be validated, the study protocol will include some or all of the phases.

If the method to be validated **is complete**, i.e. it includes the sample preparation part, then the validation study will concern the following criteria:

- Phase 1: LOD and LOQ (optional for kits only dedicated to detection), calibration function, fit, yield and robustness, verification of software calculations on quantification and detection functions
- Phase 2: inclusivity/exclusivity, practicability/quality of reagents
- Phase 3: inter-laboratory study with tests on DNA solution and artificially/naturally contaminated water samples.

If the PCR method to be validated **does not include the** sample preparation, then the study will concern the following criteria:

- Phase 1: LOD, LOQ (optional for kits only dedicated to detection), calibration function, fit, verification of software calculations on quantification and detection functions
- Phase 2 (unchanged): inclusivity/exclusivity, practicability/quality of reagents
- Phase 3: interlaboratory study with tests solely on DNA solution (no bacteria-laced samples)

If the PCR method to be validated can be used on several **thermalcyclers**, then:

- the supplier must set the list of commercial thermal cyclers qualified for its use,
- the validation study must comprise as many tests on LOD, LOQ (optional for kits only dedicated to detection), fit and calibration function (in phase 1 of the study) as there are qualified thermalcyclers.

Change requests are subject to approval by the Technical Board, which will rule on the major or minor status of the change. The supplier must seek the opinion of an expert laboratory. This opinion will be presented to the Technical Board.

If major changes are made to the validated method (extraction step, amplification phase and quantification phase), refer to the table below for the minimum number of performance criteria that must be revalidated:

Change	Performance criteria to be revalidated
Extraction phase	Yield Robustness
Amplification phase (reaction mixture - excluding probes / primers, thermal cyclers)	LOD LOQ (optional for kits only dedicated to detection) Linearity Fit
Quantification and/or detection phase (change of probe / primer system / thermal profiles)	LOD LOQ (optional for kits only dedicated to detection) Linearity Fit Inclusivity, exclusivity

Phase 1 of the study (carried out by the expert laboratory)

1. Fitting the calibration and the reference material to the primary standard

Follow the instructions given in Section 11.2 of Standard NF T90-471.

The fitting will be carried out on one batch.

2. Study of the calibration function of the quantitative PCR step

2.1. Protocol for the evaluation of the calibration line

(See § 10.3.3 of Standard NF T90-471)

The following experimental plan must be followed under intermediate reproducibility conditions.

Prepare a range of *p* levels, where *p* is the number prescribed by the supplier, equal to at least 4 and at most 6, for example 25, 250, 2 500, and 25 000 genome units of *Legionella pneumophila* per reaction tube. The range is prepared from the DNA of *Legionella pneumophila* strain WDCM 00107.

The first point in the range must be equal to the quantification limit (see § 10.4 of Standard NF T90-471). At each level, make the measurements on a total number of *k* ranges, *k* being equal to at least 5.

Record the values $y_{i,j}$ obtained, and carry out the calculations following the example provided in Table 3 of the Standard, given below.

Calculate the total number of measurements, noted N according to Equation (1):

$$N = k \times p$$

Table 1 (NF T90-471) — Expression of results and calculations

Level x_i	$x_1 = LOQ_{PCR}$	$x_2 = 10LOQ_{PCR}$	$x_3 = 100LOQ_{PCR}$	$x_4 = 1000LOQ_{PCR}$	x_p	Sum
$x_i' = \log_{10} x_i$	x'_1	x'_2	x'_3	x'_4	x'_p	
$y_{i,j}$ (k repeats)	$y_{1,1}$	$y_{2,1}$	$y_{3,1}$	$y_{4,1}$	$y_{p,1}$	
	$y_{1,2}$	$y_{2,2}$	$y_{3,2}$	$y_{4,2}$	$y_{p,2}$	
	$y_{1,k}$	$y_{2,k}$	$y_{3,k}$	$y_{4,k}$	$y_{p,k}$	
$T_i = \sum_{j=1}^{k} y_{i,j}$	T_1	T_2	T_3	T_4	T_p	$T_G = \sum_{i=1}^{p} T_i$
$m_i = \frac{T_i}{k}$	m_1	m_2	m_3	m_4	m_p	
$x_i' T_i$	$x'_1 T_1$	$x'_2 T_2$	$x'_3 T_3$	$x'_4 T_4$	$x'_p T_p$	$\sum_{i=1}^{p} x'_i T_i$

where

x_i is the number of genome units of *Legionella pneumophila* per reaction tube (the values of levels x_i are given as examples),

x'_i is the decimal logarithm of x_i

$y_{i,j}$ is the value of *CT* measured at level i (i ranges from 1 to p) and rank j (j ranges from 1 to k)

k is the number of repeats per level i ($k \geq 5$)

p is the number of levels, and is greater than or equal to 4

See Annexe C of the Standard for a concrete example of these calculations.

2.2. Analysis of results

Estimation of the regression line (See § 10.3.4.1 of the Standard)

The regression line is given by Equation (2):

$$y = CT_{\text{mean}} = ax' + b.$$

Plot the coordinates (x'_1, m_1), …(x'_p, m_p) to check visually that they are all on one straight line. If this is the case, carry out the following calculations:

$$\sum_{i=1}^{p} x'_i = k\left(x'_1 + x'_2 + x'_3 + x'_4 + ... + x'_p\right), \quad (3)$$

$$\sum_{i=1}^{p} x'^{2}_i = k\left(x'^{2}_1 + x'^{2}_2 + x'^{2}_3 + x'^{2}_4 + ... + x'^{2}_p\right) \quad (4)$$

Carry out the following calculations to determine the slope *a*:

$$\text{Variance of } x_i' = \frac{\sum x_i'^2 - \frac{\left(\sum x_i'\right)^2}{N}}{N-1} \quad (5)$$

$$\text{Covariance of } x'y = \frac{\sum x_i' T_i - \frac{\sum_i x_i' \times T_G}{N}}{N-1}. \quad (6)$$

The estimated slope *a* of the straight line is given by Equation (7):

$$a = \frac{\text{covariance of } x'y}{\text{variance of } x'}.$$

Carry out the following calculations to determine the intercept *b:*

The straight line passes through the mean abscissa value $\overline{x'} = \frac{\sum x'}{N}$ and the mean ordinate value $\overline{y} = \frac{T_G}{N}$, whence = $\overline{y} = a\overline{x'} + b$ and therefore $b = \overline{y} - a\overline{x'} = \frac{T_G}{N} - a\frac{\sum x'}{N}$.

Verification of efficiency (See § 10.3.4.2 of the Standard)

The efficiency is the measure of the correct operation of the amplification.

Calculate the efficiency *e* according to Equation (8), (see also Annex C.3 of the Standard):

$$e = (10^{-\frac{1}{a}} - 1) \times 100.$$

The slope *a* must lie between –4,115 and –2,839, so that *e* lies between 75 % and 125 %.

If *a* lies outside the range stated above, the amplification system cannot be validated.

NB. The efficiency expresses the yield of the PCR reaction.

Verification of the performance of the linear regression (See § 10.3.4.3 of the Standard)

The linear regression must meet the following accuracy requirement on each level in the range (criterion combining trueness and precision):

$$E_{LIN} \leq 0{,}15 \quad (9)$$

where

E_{LIN} (expressed in Log) is the accuracy of the linearity.

To do this, carry out the calculations indicated in Table 4 of the Standard (see also Annex C.4 of the Standard).

If $E_{LIN} \leq 0{,}15$ whatever the level *i*, then linearity is confirmed for the whole domain.
If one of the values of $E_{LIN\ i}$ exceeds the critical value of 0,15, then the linear regression model cannot be accepted. In such a case, if the number of levels tested is greater than 4, then the analysis of the data may be repeated after excluding either the low level value (x_1) or the high level value (x_p) to validate, if necessary, a part of the linear domain.

Table 2 (NF T90-471) — Calculation of bias, linearity accuracies and linearity uncertainties

Estimated level x_i	x_1	x_2	x_3	x_4	x_p
x'_i theoretical	x'_1	x'_2	x'_3	x'_4	x'_p
$x'_{i,j}$	$x'_{1,1}$	$x'_{2,1}$	$x'_{3,1}$	$x'_{4,1}$	$x'_{p,1}$
	$x'_{1,2}$	$x'_{2,2}$	$x'_{3,2}$	$x'_{4,2}$	$x'_{p,2}$
	$x'_{1,3}$	$x'_{2,3}$	$x'_{3,3}$	$x'_{4,3}$	$x'_{p,3}$
	$x'_{1,4}$	$x'_{2,4}$	$x'_{3,4}$	$x'_{4,4}$	$x'_{p,4}$
	$x'_{1,k}$	$x'_{2,k}$	$x'_{3,k}$	$x'_{4,k}$	$x'_{p,k}$
$\overline{x'_i} = \frac{\sum x'_{i,j}}{k}$	$\overline{x'_1}$	$\overline{x'_2}$	$\overline{x'_3}$	$\overline{x'_4}$	$\overline{x'_p}$
$\text{Bias} = \overline{x'_i} - x'_i$	$\overline{x'_1} - x'_1$	$\overline{x'_2} - x'_2$	$\overline{x'_3} - x'_3$	$\overline{x'_4} - x'_4$	$\overline{x'_p} - x'_p$
$s'_i = \sqrt{\frac{\sum_{j=1}^{k} x'^{2}_{i,j} - \frac{\left(\sum_{j=1}^{k} x'_{i,j}\right)^2}{k}}{k-1}}$	s'_1	s'_2	s'_3	s'_4	s'_p
$E_{\text{LIN}i} = \sqrt{s'^{2}_i + (\overline{x'_i} - x'_i)^2}$	$E_{\text{LIN}1}$	$E_{\text{LIN}2}$	$E_{\text{LIN}3}$	$E_{\text{LIN}4}$	$E_{\text{LIN}p}$
$U_{\text{LIN}i} = E_{\text{LIN}i} \times t_{k-2}$	U_{LIN_1}	U_{LIN_2}	U_{LIN_3}	U_{LIN_4}	U_{LIN_p}

where

x'_i theoretical is the value determined from Equation $x'_i = \log x_i$,

$x'_{i,j}$ is the value calculated using the equation of the calibration line from the measured value $y_{i,j}$,

$\overline{x'_i}$ is the mean value of $x'_{i,j}$,

s'_i is the standard deviation of values $x'_{i,j}$ with $k-1$ degrees of freedom,

$E_{\text{LIN}i}$ is the accuracy of linearity,

$U_{\text{LIN}i}$ is the expanded linearity uncertainty,

t_{k-2} is the value read in the Student table for $k-2$ degrees of freedom with a risk of 5% (see Annex D).

NB. Inspection of the values of bias and standard deviation shows whether the deficiency of the model is due to a problem of precision (dispersion too broad) or trueness (bias too great).

3. LOD, LOQ and positivity threshold

The following changes are proposed in the experimental plan relative to the Standard:

- Use 30 independent DNA solutions,
- The values of LOD and LOQ to be verified are those declared by the supplier.

The materials used will be those described in the supplier's instructions for use.

3.1. Limit of detection of PCR

(See § 10.5 of Standard NF T90-471)

The noted limit of detection of the PCR *LOD*pcr is estimated from the smallest number of genome units generating a positive result (amplification) at the confidence threshold of 90 %, according to the laboratory's operating procedure.

Experimental plan

- Use the declared *LOD*pcr and verify it (example: *LOD*pcr = 5 copies in the well);
- Make 30 measurements according to the supplier's protocol (number of repeats used in routine), from 30 independent dilutions prepared from a solution of DNA from *Legionella pneumophila* fitted to the primary standard. These 30 measurements can be made by the expert laboratory in the same run.

Verification of *LOD*pcr: at least 90 % of the solutions must test positive.

3.2. Limit of quantification of PCR

(See § 10.4 of Standard NF T90-471)

LOQ_{PCR} is the limit of quantification of the PCR step, and LOQ_{meth} is the limit of quantification of the entire method.

The limit of quantification LOQ_{PCR}, which is non-compressible given the dispersion of the sample set (Poisson's law), is 25 genome units counted out of all the PCR tests carried out on the sample. The value of 25 is adopted in view of the confidence interval at 95 % which gives a lower limit of 17 and an upper limit of 37. This dispersion is deemed acceptable.

Thus the value of LOQ_{PCR} targeted cannot be lower than 25 GU in a single run, 15 GU in duplicate and 10 GU in triplicate.

The limit of de quantification must correspond to the first level of the calibration range.

The LOQ_{PCR} declared by the supplier must be verified. Verification of the limit of quantification involves ensuring that the accuracy for the limit of quantification (noted E_{LOQ}) is below the critical value of 0,15.

Experimental plan

Prepare *k* = 30 independent dilutions at the value of LOQ_{PCR} targeted, from a solution of DNA from *Legionella pneumophila* fitted to the primary standard.
Quantify each of these dilutions according to the usual laboratory protocol (single run, duplicate or triplicate) in intermediate reproducibility conditions (at least on different days and/or with different operators).

The standard deviation s is given by the formula (10):

$$s = \sqrt{\frac{\sum_{i=1}^{k} x_i'^2 - \frac{\left(\sum_{i=1}^{k} x_i'\right)^2}{k}}{k-1}},$$

where

x'_i is the value calculated by inverse calibration of $\log_{10}$ of the number of genome units of *Legionella pneumophila,* and

k is the number of repeat measures.

The bias is given by the formula:

$$\mathrm{Bias} = \overline{x'_i} - \log(x), \quad (11)$$

where

x is the theoretical value of LOQ_{PCR} targeted.

Calculate the accuracy for the limit of quantification, noted E_{LOQ} using the following formula:

$$E_{LOQ} = \sqrt{s^2 + \left(\overline{x'_i} - \log(x)\right)^2} \quad (12)$$

where

S is the standard deviation of values x'_i obtained from the k measurements.

If E_{LOQ} ≤ 0,15, the limit of quantification targeted is confirmed, and the LOQ_{PCR} complies with the specifications of the Standard.

3.3. Theoretical limit of quantification of the method

(See § 10.4.4 of Standard NF T90-471)

(Section not applicable when only the quantification step is tested)

The theoretical *LOQ* of the method or LOQ_{meth} (expressed in genome units per litre) is obtained using Equation (13) as follows:

$$LOQ_{\mathrm{meth}} = \frac{LOQ_{\mathrm{PCR}} \times F}{V}, \quad (13)$$

where
V is the volume of filtered sample (in litres),
F is the multiplier (genome units per well to genome units per litre).

3.4 Positivity threshold

The limit in term of Ct or Cq set by the manufacturer (or supplier) to declare the absence or non-detection (positivity threshold) will be tested by the expert laboratory compared to the LOD. The Ct or Cq values obtained during the validation of the LOD will be verified as being less than the Ct or Cq values supplied by the manufacturer. For quantification kits, a sample with a Ct or Cq value greater than the value of the intercept (intercept of the calibration function) will be considered negative.

4. Yield of the method, robustness and uncertainty of measurement

(See § 10.6 and § 10.7 of Standard NF T90-471)

4.1. Experimental plan

The yield is studied on 10 independent samples for three different matrices at two levels of contamination, namely 10 × 3 × 2 = 60 samples.

The three types of matrix are the following:
1. Control mineral water,
2. Domestic hot water,
3. Cooling tower water.

These matrices must be free of nucleic acids from *Legionella*, and artificially contaminated with a parent suspension made up from a strain of *L. pneumophila* (strain WDCM 00107).
These matrices must have first been well characterised (physico-chemical characteristics such as pH, absence of biocides, filterability, etc.) by the expert laboratory during the study.

Two levels of contamination corresponding, for example to 1 000 and 100 000 genome units per litre must be tested. The levels of contamination may be obtained from the same parent lacing suspension.

For each level of concentration and for each matrix, at least 10 samples (2 repeats per day × 5 days) independently laced, of volume between 50 mL and 1 L, must be analysed under conditions of intra-laboratory reproducibility (different days and/or different operators, etc.).

For a test at one level of contamination, it is necessary to make up a lacing suspension from isolated colonies of the strain WDCM 00107 on BCYE-L-Cysteine or GVPC agar.
The bacterial suspension must be performed from a fresh culture (3 days of culture at 37 °C).

The artificial contamination procedure must allow the measurement of the number of genome units before the steps of concentration and extraction of nucleic acids. This measurement must be made by PCR on direct lysis of the parent lacing suspension on three separate test samples. This lysis must be carried out according to the usual lysis protocol without purification.

The mean value (noted in GU/L) calculated from the three values will serve as a reference for the calculation of the yield for each level of contamination. This value will serve to determine the lacing volume that will give the level sought (1 000 or 100 000 GU/L, for example).

If the expert laboratory possesses a spectrophotometer, it can estimate the concentration of the parent lacing suspension by measuring its absorbance at 600 nm. In these conditions, the lacing volume may be estimated before carrying out the direct lysis. The direct lysis and the extractions may thus be carried out at the same time and run on the same PCR plate.
However, the lacing levels required must be adhered to and only the PCR value can demonstrate this.

Example of the operating procedure to be followed for Day 1 (to be repeated for Days 2–5):

Day 1 Prepare a parent suspension.

Titrate the parent suspension with three direct lyses
and three quantifications. Average to carry out dilutions of lacing suspension.

Dilute the suspension to obtain two load levels corresponding to 100 000 and 1 000 GU/L

Prepare four flasks per matrix of 250 mL

Contaminate two 2 flasks with 100 µL of lacing suspension to obtain 100 000 GU/L

This must be done in all three matrices

Contaminate two flasks with 100 µL of lacing suspension to obtain 1 000 GU/L

Carry out extractions on the 4 × 3 laced flasks
+ three matrix blanks (one per matrix)

Carry out quantifications after freezing on the chosen day

Repeat the experiment on five different days, consecutive or not (or in other intra-laboratory reproducibility conditions)

This yields 60 extracts derived from one lacing and 15 blanks that must test negative

Measure the concentration in number of genome units of the parent suspension by PCR on three direct lyses of the parent suspension. These lyses must be carried out according to the usual lysis protocol without purification.

The DNA extract thus obtained must be diluted so as to remove the inhibition due to the lysis reagent.

The mean value calculated from the three values (noted *A* and expressed in $\log_{10}$ GU/mL) serves as a reference to calculate the yield.

Determine from this value the lacing volume that will give the level sought (1 000 GU/L and 100 000 GU/L, for example).

The samples thus made up (three laced solutions) follow the complete measurement protocol and give results expressed in $\log_{10}$ GU per unit volume of the parent solution, noted *B*.

The quantification of the lacing solution, and the protocol for lacing and measurement, must be carried out successively and on the same day.

4.2. Estimation of yield

The calculation of yield will be made by log difference (e.g. if the lacing used 1 000 GU, or $\log_{10}(1\,000) = 3$ and the final result is 500 GU, or $\log_{10}(500) = 2{,}7$ then the yield is equal to $2{,}7 - 3 = -0{,}3$). The $\log_{10}$ difference (yield) must lie between $-0{,}6$ and $+0{,}3$ which corresponds to a yield of between 25 % and 199 %.

Section 10.6.3 of Standard NF T90-471 gives the following formula (14):

$$\log_{10}\left(Yield_{\text{Sample } x}\right) = B - A + D + \log\frac{1000}{v_{pe}},$$

where

$\log_{10}(Yield_{\text{Sample } x})$ is the decimal logarithm of the yield of sample *x*,

A is the $\log_{10}$ of the quantity of GU per unit volume of the parent suspension, reference value obtained directly after direct lysis,

v_{pe} is the volume of the inoculated lacing suspension in microliters,

B is the $\log_{10}$ of the quantity of GU per unit volume of parent solution, measured from the laced sample that has undergone the complete method,

D is the log of the dilution factor between the parent suspension and the lacing suspension, e.g. $D = 3$ for a 1/1000 dilution.

Day 1									
Results of direct lysis on parent suspension in GU /100 µL = suspension 1									
25000	24000		26000		mean:	25000	GU/100 µL		
Dilution of the parent suspension by 10 (1 mL+ 9 mL sterile water) = suspension 2									
Deduced by calculating the value of the concentration of susp1					value:	2500	GU/100 µL		
Dilution of suspension 2 by 10 (1 mL + 9 mL sterile water) = suspension 3									
Deduced by calculating the value of the concentration of susp1					value:	250	GU/100 µL		
			Results for quantification in GU/L						
	Addition of 100 µL of lacing suspension per flask containing 250 mL of matrix		Matrix 1		Matrix 2		Matrix 3		
Level 1 = 100000 GU/L	Suspension 1		80000		75000		88000		
	Suspension 1		76000		67000		67000		
Level 2 = 1000 GU/L	Suspension 3		560		670		230		
	Suspension 3		800		600		670		
			Results for yield						
	%	Log	%	Log	%	Log	%	Log	
Mean of level 1	75.5	−0,124	80	−0,097	75	−0,125	88	−0,056	
			76	−0,119	67	−0,174	67	−0,174	
Mean of level 2	58.83	−0,259	56	−0,252	67	−0,174	23	−0,638	
			80	−0,097	60	−0,222	67	−0,174	
			Mean for matrix 1		Mean for matrix 2		Mean for matrix 3		
			73		67,25		61,25		

The mean yield and the associated standard deviations must be calculated for each level and each matrix.
According to Standard NF T 90-471, the $\log_{10}$ difference (yield) must lie between −0,6 and +0,3 which corresponds to a yield of between 25 % and 199 %.

4.3. Estimation of uncertainty of measurement

The uncertainty of measurement is estimated from the bias and the standard deviation of intermediate reproducibility.

The expanded uncertainty of measurement is calculated as follows (see Section 9.8 of Standard NF T90-471):

Overall expanded uncertainty: $U_{\text{enlarged}} = 2 \times \sqrt{Yield_{\text{mean}}^2 + s^2}$ (15)

5. Verification of calculations and interpretations made by the software

5.1. Verification of calculations

The expert laboratory must present all the calculations of results (slopes, intercepts, and values recalculated by inverse calibration) carried out according to Standard NF T90-471 and by the supplier's software.

When the compliance criteria (validity of blanks) are established by the software, the calculations must be verified by the expert laboratory.

5.2. Interpretation of inhibitions

For the inhibition control, the verification will be carried out on a total inhibitor sample by metred addition of DNA from *L. pneumophila* (of the order of 1 000 genome units) and successive dilution. Whatever the level of dilution, no underestimation (within the uncertainty of measurement) validated by the software will be accepted.

Phase 2 of the study (carried out by the expert laboratory)

1. Inclusivity and exclusivity of probes and primers

The strains listed below must come from collections or clinical or environmental samples, and be identified.

1.1. Inclusivity

(See NF T90-471 - § 10.2)
The primers and probes used must give the results expected for the following species and serogroups, which have all been isolated in humans.
Carry out inclusivity tests on DNA extracts so as to obtain about 100 genome units per well.

- Inclusivity list (micro-organisms tested recognised as belonging to the **genus *Legionella***):

L. anisa	*L. erythra* 2	*L. longbeachae* 1-2	*L. sainthelensi* 1-2
L. birminghamsis	*L. feeleii* 1-2	*L. maceachernii*	*L. tucsonensis*
L. bozemanii 1-2	*L. gormanii*	*L. micdadei*	*L. wadsworthii*
L. cherrii	*L. hackeliae* 1-2	*L. oackridgensis*	
L. cincinnatiensis	*L. jordanis*	*L. parisiensis*	
L. dumofii	*L. lansingensis*	*L. pneumophila* 1-15	

- Inclusivity list (micro-organisms tested recognised as belonging to the **species *L. pneumophila***): 15 serogroups of the species.

1.2. Exclusivity

Carry out exclusivity tests on DNA extracts so as to obtain at least 10 000 genome units per well.

- Exclusivity list (micro-organisms tested recognised as **not** belonging to the **genus *Legionella* or the species *L. pneumophila***.) These strains must occur preferentially in the same ecological niches as *Legionella* and/or be phylogenetically close. The following minimal list must be tested:

Aeromonas hydrophila	*Escherichia coli*	*Pseudomonas aeruginosa*
Alcaligenes faecalis	*Flavobacterium*	*Pseudomonas fluorescens*
Bacillus subtilis	*Klebsiella oxytoca*	*Pseudomonas putida*
Burkholderia cepacia	*Listeria monocytogenes*	*Serratia marcescens*
Clostridium	*Proteus vulgaris*	*Stenotrophomonas maltophila*
Enterobacter aerogenes		

- Exclusivity list (micro-organisms tested recognised as **not** belonging **to the species *L. pneumophila***). In addition to the strains listed above, add the following nine strains:

L. micdadei	*L. bozemanii S2*	*L. jordanis*
L. dunmofii	*L. gormanii*	*L. parisiensis*
L. anisa	*L. longbeachae S1*	*L. tucsonensis*

The result of the exclusivity test must be absence of amplification profile.
The limit in term of Ct or Cq set by the manufacturer (or the supplier) to declare the absence or non-detection will be tested by the expert laboratory with respect to the LOD. For quantification kits, a sample with a Ct or Cq value greater than the value of the intercept (intercept of the calibration function) will be considered negative.

2. Practicability

A practicability study, comprising the 18 criteria given below, will be carried out.

For each of these criteria are defined the mode of communication of the criterion to the user and the mode by which the expert laboratory checks the criterion. Some criteria need communication on the packaging or instructions; others require communication on the validation certificate.

	Criterion to be checked	Communication on the criterion	Method used to check the criterion
1	Mode of packaging of items for the method	Packaging or instructions	Verification by the expert laboratory
2	Volume of reagents	Packaging or instructions	Verification by the expert laboratory
3	Conditions of storage of items (+ expiry of unopened items)	Packaging or instructions	Verification by the expert laboratory that the conditions exist
4	Usability after first use. (in particular the existence of limiting dates)	Packaging or instructions	Verification by the expert laboratory that the modalities exist
5	Specific equipment or premises needed	Instructions	Verification by the expert laboratory that the written document is accurate
6	Reagents ready to use or to be made up (in this case, existence of instructions for use)	Packaging or instructions	Verification by the expert laboratory that the written document is accurate
7	Time needed to train operator unfamiliar with the method	Report	Measured by the expert laboratory (possibility of using times recorded by collaborating laboratories) and placed in one of the following three categories: less than one day, between one day and one week, more than one week.

	Criteria to be checked	Communication on the criterion	Method used to check the criterion
8	Real handling time / Flexibility of the technique in relation to the number of samples to be analysed, their bacterial load, etc.	Report	Measured handling time
9	Time to obtain results	Report and certificate	Establishment of two cycles describing each step of the method solely in terms of time
10	Type of qualification of the operator	Report	Stated by the expert laboratory (the expert laboratory may make use of data from collaborating laboratories)
11	If available, traceability of analysis results	Instructions	Verification by the expert laboratory
12	Maintenance by the laboratory	Report	Duration and frequency
13	Minimum pipetting volume	Report	To be stated by the expert laboratory
14	Stability of reagents and ranges	Report	To be stated by the expert laboratory • Reagent • Conditions of storage • Duration and validity • Aliquoting Note : To be checked during the NF VALIDATION audit
15	UNG treatment (prevention of contamination)	Report	To be stated by the expert laboratory
16	Protection of reagents from UV	Report	To be stated by the expert laboratory
17	External quantitative check of PCR	Report	Presence to be checked by the expert laboratory
18	Check for absence of inhibitor	Report	Verification by the expert laboratory of absence of inhibitor by internal inspection or metred addition

Phase 3 of the study: inter-laboratory study

The inter-laboratory study is intended to evaluate the precision of the commercial kit.

1. Organisation of the test campaign

This is planned in three phases. A minimum of 10 participating laboratories is advised so as to guarantee at least eight interpretable results.

Phase 1. DNA extracts

Two extracts of DNA from *Legionella* are obtained from 10 species of *Legionella* in the following list:

L. micdadei	*L. bozemanii S2*
L. dunmofii	*L. gormanii*
L. anisa	*L. longbeachae S1*
L. jordanis	*L. tucsonensis*
L. parisiensis	*L. pneumophila S1*

Preparation of two samples, each at two different concentrations. The measurements will be made according to the supplier's prescriptions (*e.g. three repeats giving one result*). Two measurements must be made in order to obtain two results for each concentration.

The purpose of the DNA test is to characterise the inter-laboratory repeatability and reproducibility of the different PCR systems (primers/probe), and their trueness. Their evaluation will be possible through the mean values of the laboratories, no true or conventional value being available.

Phase 2. BACTERIAL SUSPENSIONS

From a bacterial suspension of *Legionella pneumophila*, *Legionella spp* and non-*Legionella*, the laboratories will lace a matrix free of DNA from *Legionella* common to all the participants, so as to obtain two levels of concentration and two repeats per level

The purpose of this test is to estimate the repeatability and reproducibility of the overall methods.

Phase 3. NATURALLY CONTAMINATED SAMPLE

Homogenisation of a filterable and neutralized matrix of domestic hot water naturally contaminated with *Legionella pneumophila*, with an abundant associated flora (see NF EN ISO 6222) (more than 10^4 UG/L).
This matrix must not raise any major problem linked to inhibition.
See annex A1 for consistency and stability test protocol.
Experimental plan of the test: each participant laboratory will receive two flasks to carry out two measurements in repeatability conditions.

2. Planned timeline

A timeline will be drawn up jointly with the different laboratories selected.

The shipments will be made by the expert laboratory (see annex A2).

3. Statistical exploitation of data

The purpose of this study is to ascertain whether the method to be validated is reliable. It sets out to perform the first evaluations of measurement accuracy, as defined by Standard ISO 5725, namely: “the closeness of agreement between a test result and the accepted reference value.”

In conformity with Standard ISO 5725, we take as a target the consensus value emerging from all the observed values. In practice, this is the mean of results obtained in a normal distribution of data.

Accordingly, in what follows we will no longer be dealing with accuracy, but with precision, as defined by Standard ISO 5725, namely the closeness of agreement between repeated independent test results.

The tests proposed thus consist in drawing up experimental plans designed to quantify the precision of the method, in conditions of increasing difficulty:

- Calculation of precision linked solely to the aliquoting prescribed in the method (purely theoretical approach based on Poisson’s law, which indicates the non-compressible dispersion of the data that must at least be expected),
- Measurement of precision over part of the method, the PCR system (primers / probe). This is done on DNA extracts (first pure extracts of *L. pneumophila*, then a mixture of extracts of different *Legionella*),
- Measurement of precision of the entire method on characterised bacterial suspensions (pure suspensions of *L. pneumophila*, then a mixture of pure suspensions of various *Legionella* and interferents),
- Measurement of the precision in a real situation (naturally contaminated domestic hot water).

Express the results according to Standard ISO 5725.

NB. Only those results for which all the quality controls are compliant with Standard NF T90-471 will be exploited by the expert laboratory. Exclusion of results from a laboratory must remain exceptional, and must be systematically documented (investigation at the collaborating laboratory by the organiser laboratory).

ANNEX A

Annex A1: Consistency test protocol

A consistency test must be carried out by the expert laboratory, e.g. for 10 participant laboratories, the expert laboratory will make up 30 flasks; each participant laboratory will receive two flasks, and the expert laboratory will make three measurements on three different flasks on three consecutive days to evaluate consistency and stability.

For phase 1, the consistency of the DNA tests will be evaluated by a quantitative PCR measurement on the spp system of 20 aliquots.

For phases 2 and 3, the consistency and stability of the suspension tests will be evaluated by DNA extraction and a quantitative PCR measurement on the spp system of 9 aliquots.

Annex A2: Sample shipment protocol

Expert laboratory (organiser) prepares the materials on Monday (first day of a week comprising five working days) and stores them at a temperature of 5 °C ± 3 °C.

These materials are picked up by special carrier on Tuesday morning. The carrier must guarantee a temperature of 5 °C ± 3 °C, monitored and recorded during the journey.
The delivery must take place before midday Wednesday for all the participant laboratories.

On reception, the participant laboratories must send back to the expert laboratory a reception advice note stating at least: the date and time of reception, the condition of the materials received, the temperature of the chamber and the temperature recorded during transport.

The participant laboratories must immediately store the materials received at a temperature of 5 °C ± 3 °C.
The preparations and measurements will start on Thursday morning so as to be finished on Thursday evening. The interpretation and dispatch of results on the form provided will be done according to the indications of the expert laboratory.

Phase 1. Four tubes containing 150 µL of DNA solution are sent by special carrier at 5 °C ± 3 °C. The laboratories will be advised of the arrival of the samples a few days in advance by email. The test report form and return sheets are sent by email.

Phase 2. Four flasks containing 250 mL of artificially contaminated sample are sent by special carrier at 5 °C ± 3 °C. The laboratories will be advised of the arrival of the samples a few days in advance by email. The test report form and return sheets are sent by email.

Phase 3. Shipment of two flasks containing 250 mL of DHW sample in an isothermal container at controlled temperature (5 °C ± 3 °C).

Annex A3: Test report form / return sheets

A form listing all the steps necessary to carry out the tests is sent by email before the test materials arrive. It will state the test volumes, the sample repeats to be carried out, the nature of the *Legionella* DNA-free matrix, etc.

Results sheet (Excel) for DNA test: see below

DNA test

Date received	
Storage temperature	
Date aliquoted	

spp system

Date of PCR	

n = as prescribed by supplier	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean	
Standard deviation	

***pneumophila* system**

Date of PCR	

	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean	
Standard deviation	

Results sheet (Excel) for suspension test: see below

SUSPENSION test	
Date received	
Storage temperature of suspension	
Date extracted	
Storage temperature of DNA extracts	

method	
Concentration of bacteria	
If filtered, nature of membrane	
Type of DNA extraction	
Nature of purification	
Fraction of sample plated	

spp

Date of PCR run			
	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean of samples	
Standard deviation of samples	

pneumophila

Date of PCR run			
	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean of samples	
Standard deviation of samples	

Results sheet (Excel) for naturally contaminated sample test: see below

NATURALLY CONTAMINATED SAMPLE test

Date received	
Storage temperature of suspension	
Date extracted	
Storage temperature of DNA extracts	

Method

Concentration of bacteria	
If filtered, nature of membrane	
Type of DNA extraction	
Nature of purification	
Fraction de of sample plated	

spp

Date of PCR run			
	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean of samples	
Standard deviation of samples	

pneumophila

Date of PCR run			
	Log of number of PCR copies/wells		
	n repeats	*n* repeats	*n* repeats
Estimate of sample A1			
Estimate of sample A2			
Estimate of sample B1			
Estimate of sample B2			

Mean of samples	
Standard deviation of samples	