The determination of Legionella bacteria in waters and other environmental samples (2019) – Part 3 – Method for their detection and quantification by polymerase chain reaction (qPCR) and protocol for method validation

Methods for the Examination of Waters and Associated Materials
Methods for the Examination of Waters and Associated Materials

This booklet describes two methods which cover respectively the protocols to be used to characterise and validate the method, and the detection and quantification of Legionella bacteria in water and other environmental samples.

A  Technical protocol for the characterization and validation of the method

B  Detection and quantification of Legionella spp. and/or Legionella pneumophila in water samples by specific quantitative Polymerase Chain Reaction (qPCR).

Within this series there are three separate booklets dealing with the collection and processing of waters and environmental samples for the detection of Legionella bacteria. The other booklets are:

The determination of Legionella bacteria in water and other environmental samples (2005) - Part 1 - Rationale of surveying and sampling

The determination of Legionella bacteria in water and other environmental samples (2019) - Part 2 - Culture Methods for their detection and enumeration

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as an illustrative example of the type of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.
Within this series there are separate booklets, each dealing with different topics concerning the microbiology of drinking water. Booklets include

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 3 - Practices and procedures for laboratories
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

The Microbiology of Drinking Water (2012)
Part 5 - The isolation and enumeration of enterococci by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria
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About this series
Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are seven working groups, each responsible for one section or aspect of water quality analysis. They are:

1. General principles of sampling and accuracy of results
2. Microbiological methods
3. Empirical, Inorganic and physical methods, Metals and metalloids
4. Solid substances
5. Organic impurities
6. Biological, biodegradability and inhibition methods
7. Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA’s web-page:
http://www.standingcommitteeofanalysts.co.uk/contact.html

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Rob Carter
Secretary
April 2017

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety.

These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
Detection and quantification of \textit{Legionella \textit{spp}. and/or Legionella pneumophila} in water samples by specific quantitative Polymerase Chain Reaction (qPCR). Technical protocol for the characterization and validation of the method

\textbf{A1 General}

Technical criteria and requirements described in this part of the booklet shall be used for the characterisation and for the validation (internal/third party or primary/secondary) of any newly designed or modified methods (e.g. change in PCR kit, change in the purification method, change in the composition of the kits).

Any protocol for routine application shall have been validated according to the requirements detailed in this part.

For third party validated commercial methods that fulfil the requirements given in this part, manufacturer’s instructions shall be accurately followed.

\textit{NOTE} For primary validation, all requirements stated in this part of the booklet are applicable. For method verification, (secondary validation), simplified requirements can be used for the validation of the correct implementation in the laboratory of any third party fully validated method (see Annex G of ISO 12869:2012[1]).

\textbf{A2 Inclusivity and exclusivity of probes and primers}

Primers and probes shall provide the expected results for the following species and serogroups that have all been isolated in humans.

Perform inclusivity tests on DNA extracts containing about 100 GU/PCR well.


- Inclusivity list (microorganisms to test for a \textit{L. pneumophila method}): 15 serogroups from the species.

Perform exclusivity tests on DNA extracts containing at least 10 000 GU/well.

- Exclusivity list (tested microorganisms recognized as not belonging to \textit{Legionella genus and/or being phylogenetically close}). At least the following list shall be tested: \textit{Aeromonas hydrophila, Alcaligenes faecalis, Bacillus subtilis, Burkholderia cepacia, Clostridium spp., Enterobacter aerogenes, Escherichia coli, Flavobacterium spp., Klebsiella oxytoca, Listeria monocytogenes, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Serratia marcescens, Stenotrophomonas maltophilia.}

- Exclusivity list (tested microorganisms recognized as not belonging to \textit{L. pneumophila species}): \textit{L. anisa, L. bozemanii, L. dumoffii, L. gormanii, L. jordanis, L. longbeachae, L. micdadei, L. parisiensis and L. tucsonensis.}

Bacterial suspension concentrations can be estimated using the optical density at 600 nm. (An optical density of 0.5 at 600 nm corresponds to $10^9$ CFU/ml.)
A3 Verification of the calibration function of the quantitative PCR phase

A3.1 General

Calibration cannot easily be applied to the entire method. Only calibration of quantification by real-time PCR (on a DNA range) is described hereafter. This does not exclude the possibility of applying the same calibration function characterization rules to the entire method, i.e. artificially contaminated water samples.

The calibration function shall be done with both PCR systems, i.e. Legionella spp. and L. pneumophila.

For statistical analysis, the concentrations of genome units per PCR well are expressed as decimal logarithms.

A3.2 Calibration curve verification principle

Experience has shown that the means of Ct measurements obtained for different levels of genome unit quantities (expressed as decimal logarithms) can be represented according to a linear regression model, i.e. by a linear equation such as \( y = ax' + b \).

When the line parameters have been determined, it is then possible, by using the equation of this line, to calculate the number of legionella genome units present in the sample corresponding to a particular Ct measurement.

The parameters of the calibration curve are initially determined by following the evaluation protocol described in A.3.3. A statistical analysis (see A.3.4) is then performed in order to:

a) determine the equation for the line (see A.3.4.1)
b) verify the linear regression model (see A.3.4.3)

A3.3 Calibration curve evaluation protocol

The evaluation of the calibration features shall be performed under conditions suitable for determining reproducibility (at least on different days and/or with different operators) (e.g. one range per day for 5 days).

Prepare a range of \( p \) levels of concentrations of \( L. \) pneumophila genome units [preferably prepared from the primary standard and, in any case, prepared from \( L. \) pneumophila (WDCM 00107)], \( p \) being at least equal to 4, for example, 25, 250, 2500, 25 000 genome units of \( L. \) pneumophila per PCR well. The first point in the range shall be equal to the limit of quantification (see B.4). At each level perform \( k \) repetitions of the measurement, \( k \) being at least equal to 5. Record the obtained \( y_{i,j} \) values according to the example given in Table 1.

Perform the calculations as indicated in the Table A1.
### Table A1 — Formatting of results and calculations

<table>
<thead>
<tr>
<th>Level $x_i$</th>
<th>$x'_1$</th>
<th>$x'_2$</th>
<th>$x'_3$</th>
<th>$x'_4$</th>
<th>$x'_p$</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>$= \log_{10} x_i$</td>
<td>$x'_1$</td>
<td>$x'_2$</td>
<td>$x'_3$</td>
<td>$x'_4$</td>
<td>$x'_p$</td>
<td></td>
</tr>
<tr>
<td>$Y_{i,j}$ (k repetitions)</td>
<td>$y_{1,1}$</td>
<td>$y_{2,1}$</td>
<td>$y_{3,1}$</td>
<td>$y_{4,1}$</td>
<td>$y_{p,1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$y_{1,2}$</td>
<td>$y_{2,2}$</td>
<td>$y_{3,2}$</td>
<td>$y_{4,2}$</td>
<td>$y_{p,2}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$y_{1,k}$</td>
<td>$y_{2,k}$</td>
<td>$y_{3,k}$</td>
<td>$y_{4,k}$</td>
<td>$y_{p,k}$</td>
<td></td>
</tr>
<tr>
<td>$T_i = \sum_{j=1}^{k} y_{i,j}$</td>
<td>$T_1$</td>
<td>$T_2$</td>
<td>$T_3$</td>
<td>$T_4$</td>
<td>$T_p$</td>
<td>$T_G = \sum_{i=1}^{p} T_i$</td>
</tr>
<tr>
<td>$m_i = \frac{T_i}{k}$</td>
<td>$m_1$</td>
<td>$m_2$</td>
<td>$m_3$</td>
<td>$m_4$</td>
<td>$m_p$</td>
<td></td>
</tr>
<tr>
<td>$x'_i T_i$</td>
<td>$x'_1 T_1$</td>
<td>$x'_2 T_2$</td>
<td>$x'_3 T_3$</td>
<td>$x'_i T_i$</td>
<td>$\sum_{i=1}^{p} x'_i T_i$</td>
<td></td>
</tr>
</tbody>
</table>

$x$ number of *L. pneumophila* genome units per PCR well (the values of $x$ levels are given as examples)

$x'_i$ logarithm of $x_i$

$y_{i,j}$ Ct value measurement at level $i$ ($i = 1 \ldots p$) and row $j$ ($j = 1 \ldots k$)

$k$ number of repetitions per level $i$ ($k \geq 5$)

$p$ number of levels ($p \geq 4$)

---

Calculate the total number of measurements noted $N$ according to Formula (1):

$$N = kp \quad (1)$$


#### A3.4 Analysis of the results

#### A3.4.1 Estimation of the regression curve

The regression curve is given by Formula (2):

$$y = \mu_{C_t} = ax' + b \quad (2)$$

where $\mu_{C_t}$ is the average Ct value.

Plot the points with coordinates ($x'_1, m_1$), ($x'_p, m_p$) on a graph in order to visually verify their alignment along the curve. If this examination is satisfactory, proceed to the following calculations:

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

$$\sum_{i=1}^{p} x'_i^2 = k \left( x'_1^2 + x'_2^2 + x'_3^2 + x'_4^2 + \ldots + x'_p^2 \right) \quad (4)$$
Proceed to the following calculations in order to determine the slope \( a \). The variance of \( x'_i \), \( Vx'_i \), is given by

\[
Vx'_i = \frac{\sum x'_i^2 - \left( \frac{\sum x'_i}{N} \right)^2}{N-1}
\]  

and the covariance of \( x'y \), \( \sigma_{xy} \) by

\[
\sigma_{xy} = \frac{\sum x'_i T_i - \left( \frac{\sum x'_i T_i}{N} \right) }{N-1}
\]  

The estimation of the slope \( a \) is given by Formula (7):

\[
a = \frac{\sigma_{xy}}{Vx'_i}
\]  

Proceed to the following calculations in order to fix the intercept point \( b \). The curve passes through the average point whose coordinates are, on the abscissa

\[
\bar{x}' = \frac{\sum x'}{N}
\]

and on the ordinate

\[
\bar{y} = \frac{T_G}{N}
\]

Consequently,

\[
\bar{y} = ax' + b
\]

and therefore

\[
b = \bar{y} - ax' = \frac{T_G}{N} - a \frac{\sum x'}{N}
\]

A3.4.2 Estimate and verification of the efficiency

Efficiency assesses the yield of the PCR reaction. Efficiency \( e \) is calculated using Formula (8):

\[
e = (10^{-1/a} - 1) \times 100
\]  

Efficiency shall have a value between 75 % and 125 %. Consequently, the value of the slope, \( a \), shall be between \(-4,115\) and \(-2,839\). If \( a \) is outside the range, the amplification system shall not be validated.
A3.4.3 Verification of the linear regression performance

The linear regression shall satisfy the following accuracy requirement for each level of the standard curve (criteria that include the bias and the precision) as follows:

\[ E_{\text{lin}} \leq 0.15 \]  \hspace{1cm} (9)

where \( E_{\text{lin}} \), expressed as a decimal logarithm, is the accuracy of linearity.

To do this, proceed to the calculations given in Table A2.

If \( E_{\text{lin}} \leq 0.15 \), whatever the level of \( i \), the linearity is then verified for the whole domain.

If one of the \( E_{\text{lin},i} \) values is above the critical value of 0.15log\(_{10}\)unit, the model shall not be validated. In this case, if more than four levels were tested, the data analysis shall be rerun with either the lowest level value (\( x_i \)) or the highest level value (\( x_p \)) removed, in order to validate a part of the linear range.

NOTE Examination of the bias values and standard deviations shows whether the model error is caused by a precision issue (\( s' \); too high) or a bias issue (\( x_i^' - x_i \); too high).

Table A2 — Bias, precision, accuracy and uncertainty of linearity calculations

<table>
<thead>
<tr>
<th>Estimated ( x_i )/level</th>
<th>( x_1 )</th>
<th>( x_2 )</th>
<th>( x_3 )</th>
<th>( x_4 )</th>
<th>( x_p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical ( x_i )/level</td>
<td>( x_{i,1} )</td>
<td>( x_{i,2} )</td>
<td>( x_{i,3} )</td>
<td>( x_{i,4} )</td>
<td>( x_{i,5} )</td>
</tr>
</tbody>
</table>

\[ x_i = \frac{\sum x_{i,j}}{k} \]

\[ s_i = \sqrt{\frac{\sum_{j=1}^{k} x_{i,j}^2 - \left( \frac{\sum_{j=1}^{k} x_{i,j} \cdot x_{i,j}}{k} \right)^2}{k-1}} \]

\[ E_{\text{lin},i} = \sqrt{s_i^2 + \left( x_i^' - x_i \right)^2} \]

\[ U_{\text{lin},i} \]

Theoretical \( x_i \) value calculated using the following equation \( x_i^1 = \log x_i \)

\( x_{i,j} \) value calculated using the standard curve from the measurement value \( y_{i,j} \)

\( \bar{x}_i \); average of \( x_{i,j} \)

\( s_i \); standard deviation of values \( x_{i,j} \) with \( k-1 \) degrees of freedom

\( E_{\text{lin}} \); accuracy of linearity

\( U_{\text{lin}} \); expanded uncertainty of linearity

\( t_{k-2} \); value given by the Student table for \( k - 2 \) degrees of freedom at a risk of 5\% (see Annex D ISO 12869:2012)

A3.5 Use of the calibration curve

For each measurement \( y = Ct \) of a sample, use the standard curve formula to obtain \( x' \) by inverse calibration:

\[ x' = \frac{y - b}{a} \]  \hspace{1cm} (10)
Calculate the average of \( x', \overline{x'} \), and the associated standard deviations, according to the formula specified in Table 6.

NOTE If the associated standard deviation, \( s' \), is greater than 0.15, the uncertainty of the sample measurement is greater than the uncertainty estimated during the initial method characterization.

By antilog transformation, express the result as \( x \) GU/litre as per Formula (11):

\[
x = 10^{\overline{x'}}
\]  

(11)

A4 Verification of the PCR limit of quantification, LQ_qPCR

A4.1 Principle

The lowest acceptable limit of quantification is 25 GU (1.40log_{10} unit) due to the sampling distribution (Poisson distribution) over all the tests performed on the sample. The limit of quantification shall correspond to the first level of the calibration range. The quantification limit is verified if the lack of accuracy at the quantification limit, \( E_{LQ} \), is less than or equal to the critical value of 0.15log_{10}unit.

NOTE: The 0.15log_{10} unit value comes from experimental data.

A4.2 Experimental design

Prepare \( k \) separate dilutions (\( k \geq 10 \)) at the targeted LQ_qPCR value from a DNA solution of \( L. pneumophila \) derived from the primary standard (see A.2). Quantify each dilution according to usual laboratory protocol (single, duplicate or triplicate) under these intermediate precision conditions (at least on different days and/or by different operators). The targeted LQ_qPCR value may not be less than 25 GU for a single measurement, 15 GU for duplicate and 10 GU for triplicate measurements.

A4.3 Analysis of results

Calculate the standard deviation for the \( x'_i \) values obtained via inverse calibration from the \( k \) measurements:

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

(12)

Where

\( x'_i \) is the decimal logarithm of the number of genome units of \( L. pneumophila \), calculated by inverse calibration, from the \( C_l \) values and the calibration curve equation;

\( k \) is the number of measurements.

Calculate the bias using Formula (13):
where $x$ is the theoretical value of the targeted LQ<sub>qPCR</sub>.

Calculate the accuracy at the limit of quantification, $E_{[LQ]}$, using Formula (14):

$$E_{[LQ]} = \sqrt{s^2 + \left(\bar{x}_i - \log_{10}(x)\right)^2}$$  \hspace{1cm} (14)

Where

$E_{[LQ]}$ is the accuracy at the limit of quantification;

$s$ is the standard deviation of the $x_i$ values obtained from the $k$ measurements.

If $E_{[LQ]} \leq 0.15$, the targeted limit of quantification is verified. Otherwise look for the causes (values too low, outliers, etc.).

Calculate the uncertainty at the limit of quantification ($U_{[LQ]}$) using Formula (15):

$$U_{[LQ]} = E_{[LQ]} t_{[k-1]}$$  \hspace{1cm} (15)

Where

$U_{[LQ]}$ is the uncertainty at the limit of quantification;

$t_{[k-1]}$ is the Student table value (at 5 % risk, for $k - 1$ degrees of freedom).

An example of LQ<sub>qPCR</sub> verification for a targeted LQ<sub>PCR</sub> at 25 GU, with 10 measurements, is given in Table A3.

**Table A3. Example of LQ<sub>qPCR</sub> verification**

<table>
<thead>
<tr>
<th>Test No.</th>
<th>$x_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.498</td>
</tr>
<tr>
<td>2</td>
<td>1.577</td>
</tr>
<tr>
<td>3</td>
<td>1.461</td>
</tr>
<tr>
<td>4</td>
<td>1.480</td>
</tr>
<tr>
<td>5</td>
<td>1.515</td>
</tr>
<tr>
<td>6</td>
<td>1.531</td>
</tr>
<tr>
<td>7</td>
<td>1.442</td>
</tr>
<tr>
<td>8</td>
<td>1.422</td>
</tr>
<tr>
<td>9</td>
<td>1.547</td>
</tr>
<tr>
<td>10</td>
<td>1.499</td>
</tr>
<tr>
<td>$\bar{x}_i$</td>
<td>1.497</td>
</tr>
<tr>
<td>Bias</td>
<td>0.099</td>
</tr>
<tr>
<td>$S$</td>
<td>0.048</td>
</tr>
<tr>
<td>$E_{[LQ]}$</td>
<td>0.110</td>
</tr>
<tr>
<td>$U_{[LQ]}$</td>
<td>0.249</td>
</tr>
</tbody>
</table>

In the example in Table 3, $E_{[LQ]} = 0.11$, therefore $E_{[LQ]} \leq 0.15$, and the limit of quantification at 25 GU is validated.
A4.4 Theoretical limit of quantification of the whole method

The theoretical LQ of the method or LQ\textsubscript{meth} (expressed in genome units per litre) is obtained using Formula (16):

\[
LQ\textsubscript{meth} = \frac{LQ\textsubscript{PCR} F}{V}
\]  \hspace{1cm} (16)

Where:

- \( F \) is a conversion factor of No. of genome units per well to No. of genome units per litre;
- \( V \) is the filtered volume of sample.

NOTE This LQ\textsubscript{meth} does not take into account the recovery inherent to the preparatory phases.

A5 Verification of the PCR limit of detection (LD\textsubscript{qPCR})

The limit of detection corresponds to the smallest number of genome units that provides a PCR positive result at the 90 % threshold.

Check that at least 90 % of the results for the targeted LD\textsubscript{qPCR} value are positive (e.g. 5 GU/PCR well) so as to limit the number of tests. Take at least 10 measurements for the chosen LD\textsubscript{qPCR} from 10 separate dilutions prepared from a DNA solution of \textit{L. pneumophila} connected to the primary standard (see A.2).

A6 Recovery method

A6.1 Principle

The recovery study shall be carried out on sterile water samples (without \textit{Legionella} DNA) that have been artificially contaminated with dilutions of a mother suspension formed from a strain of \textit{L. pneumophila} (WDCM 00107).

At least two spiked levels (dilutions) shall be tested corresponding, for example, to 1 000 GU/l and 100 000 GU/l. These two levels shall come from different replicate serial dilutions derived from the same mother suspension.

For each level of concentration, at least 10 separate spiked samples with volumes between 100 ml and 1 l shall be analysed under intermediate precision conditions (over several days, by several technicians, etc.).

Calculate the recovery by logarithm difference. Recovery shall have a value between \( -0.6 \log_{10}\) unit and \(+0.3 \log_{10}\) unit.

A6.2 Protocol

To perform a series of tests, create a mother suspension from colonies of \textit{L. pneumophila} (WDCM 00107), then aseptically inoculate the \textit{L. pneumophila} colonies (e.g. five), that are less than 72 h old, in a tube containing 2 ml of tryptone salt in order to obtain a mother suspension that theoretically contains \(10^9\) GU/ml. It is advisable to verify the concentration of the mother suspension by measuring its optical density at 600 nm. An optical density of 0.5
at 600 nm corresponds to a *Legionella* spp. concentration of $10^9$ organisms/ml. Vigorously homogenize the mother suspension for at least 30 s.

Measure the concentration of genome units in the mother suspension by PCR from three direct lyses of the mother suspension: simultaneously apply the lysis protocol of the method (lysis solution and physical conditions such as temperature, time, shaking) to three test portions of the mother suspension.

The minimum test portion volume is 100 μl introduced into the normal volume of lysis solution (the ratio between the volume of the lysis solution and the test portion volume shall be at least 3). At the end of the lysis, the three unpurified DNA extracts thus obtained shall be, if necessary, diluted so as to remove any lysis reagent-associated PCR inhibition and then, quantified by PCR.

Calculate the average value, $A$, expressed in GU/ml, from the three logarithmic values obtained. This $A$ value acts as a reference for the recovery calculation.

Simultaneously create spiked suspensions, from the mother suspension, so as to obtain the targeted concentration levels, i.e. prepare a range of dilutions from the mother suspension (e.g. $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$ designated $d_1$, $d_2$, $d_3$, $d_4$, and $d_5$, respectively). The serial dilutions shall be carried out at $10^{-1}$ in tryptone salt at 9 ml tryptone salt to 1 ml bacterial suspension. Each tube shall be homogenized by mechanical shaking (approximately 10 s). The dilution levels $10^{-3}$ ($d_3$) and $10^{-5}$ ($d_5$) correspond, respectively, to approximately $10^6$ GU/ml and $10^4$ GU/ml. Prepare two spiked samples by inoculating a minimum volume of 100 μl (designated $V_{pe}$), of the two chosen dilutions, e.g. $d_3$ or $d_5$, enabling the respective quantities of $10^5$ GU and $10^3$ GU to be obtained in the filtered volume (0.1 l to 1 l), in this case. The two spiked samples thus obtained (two different levels) shall follow the full measurement protocol (filtration, extraction, and measurement) and shall lead to results $B$, expressed as a decimal logarithm per sample.

The PCR quantification of the mother suspension and the spiked samples shall be carried out on the same day, in the same amplification series.

### A6.3 Calculations

The recovery calculation for a sample is obtained by Formula (21):

$$\log_{10} \eta_x = B - A + D + \log_{10} \frac{1000}{V_{pe}}$$

(21)

Where:

- $\log_{10} \eta_x$ is the decimal logarithm of recovery for sample $x$;
- $A$ is the reference value for the concentration of the mother suspension, expressed as a decimal logarithm of the number of genome units per millilitre;
- $V_{pe}$ is the volume of the spiking suspension, in microlitres, μl;
- $s$ the value measured from the spiked sample, expressed as a decimal logarithm of the number of genome units per sample;
$D$ is the decimal logarithm of the dilution factor between the mother suspension and the spiked suspension, e.g. $D$ is 3 for a $10^{-3}$ dilution.

The procedure described above shall be carried out at least 10 times for each spiked level under intermediate precision conditions (at least on different days and/or by different operators). Calculate the average recovery and its standard deviation from the 10 individual recovery values obtained.

The average recovery per level shall have a value between $-0.6 \log_{10}$unit and $+0.3 \log_{10}$unit. These values may be the first values used for the introduction of control charts.

If the value obtained is not within the expected limits, the causes shall be investigated. See example in Annex E of ISO 12869:2019 [1].

A7 Robustness

In this instance robustness is determined through the characterization of the matrix effect. Recovery shall not be substantially affected by the type of matrix to be analysed.

To do this, the recovery for each type of matrix to be tested shall be determined by the laboratory (e.g. cooling tower water, potable water, surface water, waste water) and followed over time (optional control charts). Follow the protocol described in A.6, by replacing the sterile water with the $L. pneumophila$ free matrix (the minimum filtered volume shall be 100 ml). The acceptable limits are the same as those in A.6.

A8 Measurement uncertainty of the whole method

The uncertainty measurement of the whole method encompasses both accuracy and intermediate precision. The approach described in this Technical Specification is based on the analysis of the recovery values.

The bias is estimated by the average recovery value for all the matrices (A.6 and A.7). The precision is estimated through the recovery variance, using all the values obtained during the initial validation of the method (A.6), the robustness study (A.7), and the monitoring of recovery over time (B.10.4).

Only the lysis recovery is not included in the uncertainty evaluation. It is recommended that the laboratory lysis protocol be compared with different commercial lysis protocols in order to check the lack of bias of this stage.

A recovery measurement is obtained from two PCR measurements (global method and direct lysis). The uncertainty evaluation is therefore overestimated.

Proceed to the calculations stated in Table 4 (see example in Annex F of PD ISO 12869:2019[1]).

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Matrix</th>
<th>Level tested</th>
<th>Sample recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x=1...n$</td>
<td>Sterile water</td>
<td>Level 1 (e.g. 1000GU/L)</td>
<td>$\eta_x$</td>
</tr>
<tr>
<td>where n is the total number of samples</td>
<td>Hot sanitary water</td>
<td>Level 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level 2</td>
<td></td>
</tr>
</tbody>
</table>
for all matrices and all levels together

<table>
<thead>
<tr>
<th></th>
<th>Air cooling water</th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etc.</td>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
</tbody>
</table>

Average recovery ($\bar{\eta}_x$)

$$\frac{\sum^n_{x=1} \eta_x}{n}$$

Variance ($s^2$)

$$s^2 = \frac{\sum^n_{x=1} \eta_x^2 - \left(\sum^n_{x=1} \eta_x\right)^2/n}{n-1}$$

Overall expanded uncertainty, $U_{overall}$

$$U_{overall} = 2 \times \sqrt{\bar{\eta}_x^2 + s^2}$$

A9 References

B Detection and quantification of \textit{Legionella} spp. including \textit{Legionella pneumophila} in water samples by specific quantitative Polymerase Chain Reaction (qPCR).

B1 Introduction

\textit{Legionella} species, the cause of legionellosis, are aquatic bacteria that are widespread in nature and occur in water over a wide temperature range but most frequently between temperatures of 30-45 °C. Their tolerance to relatively high temperatures facilitates the colonisation of anthropogenic water systems such as for example cooling towers, hot and cold water systems and spa pools\cite{1}. Legionella species are typically transmitted from these systems via aerosols.

Water samples may be examined for Legionella species as part of surveillance programs designed to verifying risk and the effectiveness of control measures and during public health epidemiological investigations, or, or in order to validate new biocide treatment or other control methods\cite{1}. Routine sampling should also be carried out based on Legionella Risk Assessments following national requirements/guidance\cite{2}.

The numbers of legionellae are often low and as a result, it is usually necessary to concentrate the microbiota from water samples before their analysis. However, in outbreak investigations, when potential sources may show high numbers of legionellae and be heavily contaminated with other bacteria, samples should be examined with and without concentration stages being used. Concentration can be achieved using filtration or centrifugation techniques, or a combination of both.

The culture method remains the "gold standard" for detecting \textit{Legionella} from environmental sources \cite{8}, \cite{10}, \cite{11} & \cite{12}. Culture, requires up to 10 days to complete, potentially delaying identification of \textit{Legionella} sources and implementation of control measures. The culture method lacks the sensitivity of rapid PCR methods and some species of the genus \textit{Legionella} are unable to grow on the selective media or at the recommended culture temperature. Furthermore, viable but not culturable (VBNC) cells that may pose a public health risk are not detected by culture method but can be by some new rapid methods.

In order to overcome the drawbacks of culture, molecular methods, and mainly polymerase chain reaction (PCR), have been developed. PCR is extremely sensitive and only requires a few hours to complete, providing an extremely powerful screening tool for the rapid detection of \textit{Legionella} in environmental samples. Nevertheless, traditional PCR or real time PCR does not permit to distinguish between living and dead cells, although dormant cells, viable but non culturable cells (VBNC) and \textit{Legionella} living within amoeba can be easily detected and quantified. PCR also offers the potential of detecting \textit{Legionella} when culture methods fail due to contamination of the sample with other microbial flora. This is frequently encountered in water systems that have lost microbial control (cross reference with culture method section of the blue book guide). Rapid characterisation and enumeration of samples by PCR can be an important advantage when outbreaks or clusters of Legionnaires’ disease occur, helping to identify or rule out sources in a timely manner and improving the management and the containment\cite{7}.

B2 Scope

This document describes a molecular based method for the detection and quantification of legionellae in water samples, as collected using procedures described elsewhere \cite{1} & \cite{3}. It specifies general methodology and quality control requirements.
Technical details specified in this document are given for information only. Any other technical solutions using commercially available kits or in-house developed methods complying with the performance requirements are suitable.

This methodology can be used to identify and quantify *Legionella* to the genus level alone, or to the *L. pneumophila* species level, depending on laboratory protocol and regulations.

The information in this document is intended to be applied in the bacteriological investigation of all types of water. However, some additives, e.g. chemicals used for water treatment, the nature and/or content of suspended matter and/or accompanying microbiota can interfere with the method and affect its sensitivity.

The results are expressed as the number of genomic units of *Legionella* spp. and/or *L. pneumophila* per litre or per volume of sample.

Users wishing to employ these methods should verify its performance under their own laboratory conditions as described in Method A.

**B3 Definitions**

*16s rRNA* - 16s ribosomal ribonucleic acid

*Amplification (DNA replication)* - increase of DNA fragments or amplicons as a result of the PCR reaction.

*Amplification series* - set of PCR amplifications run while using the same PCR reagent batches, same materials, and same instruments.

*Ct value (threshold cycle)* - number of PCR cycles (denaturation and amplification) required to replicate the DNA copies originally present in the sample, so that the concentration of DNA exceeds the detection limit.

NOTE The Ct value is the intercept of the line that represents the DNA concentration of a sample with the fluorescent base line.

*Deoxyribonucleic acid (DNA)* - genetic material of living organisms consisting of generic and very specific parts.

*Detection limit of the qPCR (LD<sub>qPCR</sub>)* - lowest number of genomic units that give a positive result in the qPCR with 90 % confidence.

*Genomic unit (GU)* - a single copy of the target organism genomic DNA.

*Legionella* - a genus consisting of approximately 60 species of Gram-negative bacteria. In terms of PCR Legionella can be defined by DNA sequences of genes encoding its specific 16S rRNA.

*Legionella pneumophila DNA primary standard* - calibrated DNA solution of *L. pneumophila* (WDCM 00107) with a known quantity of genomic units and an associated uncertainty.

NOTE 1: The standard is used to adjust the working calibration DNA solutions.

NOTE 2: For the WDCM catalogue, see [9].
Macrophage infectivity potentiator gene (mip gene) - gene present in Legionella spp. which is essential for the infection of the host (protozoa) and macrophages (humans).

NOTE: The unique base sequence of the mip gene of L. pneumophila can be used for the design of the primer and probe sequences for the specific qPCR detection of L. pneumophila.

PCR inhibition control - calibrated DNA that is required to be co-amplified with the sample DNA extract. This DNA can be amplified by separate primers or using the primers needed for Legionella spp. or L. pneumophila detection.

NOTE: The PCR inhibition control should reveal any inhibitor presence in the sample DNA extract.

PCR product (PCR amplicon) - DNA that is synthesized by the PCR.

Polymerase chain reaction (PCR) - enzymatic procedure whereby a specific DNA fragment is replicated by a cyclical iterated process of denaturation, annealing of specific primers and DNA synthesis.

Primer (forward and reverse primers) - single-strand DNA fragment (oligonucleotide) that serves as a template for specific DNA replication.

NOTE: The choice of the DNA sequences of both the forward and reverse primers determines which DNA fragment is replicated. The length of the primer varies from 15 to 30 nucleotides.

Probe - single-stranded DNA fragment, targeting a specific sequence, labelled with a fluorophore that can be detected in the real-time PCR device

Quantification limit of the qPCR (LQ_{qPCR}) - lowest number of genomic units that can be quantified with an accuracy less than or equal to 0,15 log_{10} unit

Quantitative PCR (qPCR) - formation of specific DNA fragments which are highlighted by a labelled fluorescent probe and monitored in real time.

NOTE: The intensity of the fluorescence is a measure of the amount of amplicons. By comparison with calibration curve, the initial concentration of the DNA target can be determined.

Real-time PCR thermocycler - Device used for amplification by PCR which, after each cycle of polymerization, records a fluorescent signal which is proportional to the amount of amplification product (genomic units).

Recovery - efficiency of the DNA extraction.

Reference material - ready-to-use calibrated DNA solution connected to the L. pneumophila DNA primary standard

NOTE: The reference material shall be processed in each PCR run to check the accuracy of the qPCR.

Taq DNA polymerase - enzyme obtained from the bacterium Thermus aquaticus that lives in hot springs and geysers.
NOTE 1: This thermo-stable polymerase is used for the DNA synthesis in the PCR.
NOTE 2: The use of hot-start polymerase is possible to avoid false-positive results.

**Working calibration solutions** - *L. pneumophila* (WDCM 00107) DNA calibrated solutions, compared to the *L. pneumophila* DNA primary standard, used to establish the calibration curve.

NOTE: The procedure is specified in 10.2

**B4 Principle**

The detection and quantification of *Legionella* spp. and *Legionella pneumophila* by qPCR is carried out in three phases:

— concentration of water samples by filtration (or alternatively by centrifugation);
— DNA extraction from the filter (or concentrate);
— amplification, detection and quantification of one or more specific DNA sequences belonging to the *Legionella* genus and/or *L. pneumophila* by real-time qPCR;

**B5 Limitations**

Sensitivity of qPCR for bacterial quantification in water samples will be dependent on the type of water to be tested. The presence of some chemicals, biocides, suspension matter (organic matter) or large concentrations of microbiota can affect negatively the Limits of Detection and Quantification of the method. Inhibition of the reaction due the presence of interfering material can be solved by diluting the original sample which will produce a higher Limit of Quantification.

Interpretation of the results can be difficult and advice of an expert is recommended. Moreover, since traditional qPCR does not discriminate between live or dead cells its result is not directly comparable with culture.

The significance of a qPCR positive detection/culture negative sample is uncertain in a risk management perspective, although it may indicate that the site was colonised with *Legionella* (at some stage) and that there is adequate disinfection (the PCR detects DNA from dead cells which are non culturable).

**B6 Health and safety**

**WARNING - Species of Legionella are pathogenic**

Risk of harm is caused by the inhalation of aerosolised disease causing strains of *Legionella* bacteria it is therefore advisable to assess all techniques for their ability to produce aerosols.

All samples submitted for *Legionella* analysis should therefore be regarded as potentially contaminated with bacteria classified as “Hazard Group 2” and handled with strict adherence to the general safety precautions described for work at Containment Level 2 (CL2), to include minimising exposure to aerosols at all times.

Based on the risk assessment of procedures undertaken during it may be necessary to carry out the examination in a microbiological safety cabinet. However, experience has determined
that Legionella spp. can be handled safety by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to containment level two.

Media, reagents and bacteria used in this method are covered by regulations and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere in this series[5].

**B7 General Testing conditions**

The principles to be applied are as follows:

— use of disposables compatible with PCR methods is preferred;
— a procedure for eliminating DNA traces shall be implemented in event of accidental contamination of the premises or apparatus;
— regular quality controls checks shall be used to demonstrate the effectiveness of maintenance procedures with the objective of ensuring that there is no contaminating Legionella DNA (see Section D10).

The user should always pay attention to the following:

- Use gloves as well as sterile pipette tips with filters or positive displacement pipettes and tips.
- All materials and media possibly containing the tested pathogen should be autoclaved for 20 min at 120°C after use.
- Store and extract positive materials (specimens, positive controls, calibration standards and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.

**B7.1 Staff**

Most molecular tests require highly skilled and well-trained staff. To achieve this all staff must be trained and then deemed competent prior to starting testing samples in the laboratory. Furthermore, it is advisable to assess the competency of the staff on an on-going basis using either external or internal quality control programs.

Once this has been completed the laboratory manager should formally approve the staff member competent to conduct testing. This training should be recorded and documented.

The staff shall wear separate laboratory coats for microbiology activities involving cultures and molecular biology activities. Any gloves used shall be disposable and talc free.

Laboratory coats shall be changed between the areas of low DNA concentration (pre-amplification) and the areas of high Legionella DNA concentration (post-amplification). When laboratory coats are not disposable, then they shall be periodically cleaned and replaced. Only duly equipped staff shall have access to the specific rooms where these tests are run.
B7.2 Premises

PCR is a sensitive detection method. Aerosols, dust, and other particles are carriers of contaminating DNA.

Where possible PCR facilities should be organised into up to four discrete areas/rooms:

a) An area for the concentration of samples and DNA extraction;

b) An area for the preparation of PCR reagents (reaction mixtures);

c) An area for amplification;

d) A product analysis area where post-PCR manipulations are performed e.g. agarose gel electrophoresis of products, PCR-ELISA detection system. This is a contaminated area and therefore no reagents, equipment, laboratory coats etc. from this room should be used in any of the other PCR areas.

Requirements may vary with the assay format and platform. For example, for real-time PCRs only 3 areas may be required as post-PCR analysis is not required. However, for nested PCR assays, the additional steps require that four rooms/areas are available. Workflow between these rooms/areas must be unidirectional i.e. from clean areas to contaminated areas, but not from contaminated areas to clean labs. Dedicated laboratory coats shall be supplied for each area and gloves shall be changed between areas. Staff will have to leave product analysis areas and go back to the earlier rooms eventually. It is here that rigid adherence to good practise is most essential. Coats, gloves and any other personal protective equipment should be changed, and hands washed. No working materials can be brought back to earlier stages, not even notebooks or pens or memory sticks.

If automated machines are used, then certain activities can be grouped together in the same area. In all cases, check that there is no contamination (see Section 10).

Regardless of the amplicon detection and amplification system used, no tube shall be opened after amplification in areas a), b), and c).

B7.3 Apparatus

B7.3.1 PCR hood/cabinet

Ideally equipped with UV lamps to ensure decontamination of equipment used.

B7.3.2 Centrifuge

Centrifuge requirements will vary depending on the qPCR protocol followed. It is advisable to have a bench top centrifuge for large volumes and a microcentrifuge for 1.5 ml or smaller tubes.

B7.3.3 Vortex
B7.3.4 Water bath
B7.3.5 Real-time PCR thermocycler
B7.3.6 Membrane filtration apparatus

Suitable for filtering water samples of volumes up to 1.0 Litre. This apparatus normally includes a filter stand and funnel and should withstand autoclaving (alternatively sterile disposal funnels are available). The filter diameter may vary between 47-142 mm. Larger apparatus are usually constructed of stainless steel.

B7.3.7 Micropipette

To avoid cross-contamination by aerosols, use tips with hydrophobic filters and/or positive displacement micropipettes. Use a separate set of micropipettes for each area of activity.

B7.4 Consumables

All consumable materials (including tips and tubes) used should be free from DNA, RNA and DNase or if not then *Legionella* DNA free as well as PCR inhibitors free.

Apart from the concentration phase, it is important to avoid the apparatus coming into contact with the water sample so as to prevent cross-contamination. Single-use disposables are recommended.

The quality control shall be used to confirm the effectiveness of the decontamination protocols. Wherever possible, use consumables of “molecular biology” quality.

Careful consideration should be given to the apparatus and consumables specified in 7.3 to 7.4.

B7.4.1 Membrane filters

Membrane filters shall be made of polycarbonate, nylon or any other compound with a low capacity for adsorption of protein or DNA, with a nominal porosity of 0.45 μm or less. Do not use a membrane containing cellulose.

B7.5 Reagents

B7.5.1 General

All reagents used shall be sterile, free from nucleases and PCR inhibitors; ideally they should be DNA free or if not then *Legionella* DNA free.

Whenever possible, all reagents shall be dispensed in appropriate volumes so as to avoid reusing the aliquots. This improves the repeatability of the method. Suitable procedures shall be used to ensure traceability of all reagents.

Follow suppliers’ recommendations for storage and handling of reagents.

B7.5.2 PCR reagents
A PCR reaction mixture generally contains the components indicated in Table B1. Ready to use formulations of PCR reaction mixtures are available from a number of commercial suppliers. The reaction volumes handled during PCR tests are usually between 1 μl and 100 μl. To increase PCR repeatability while decreasing the uncertainty associated with small volumes, sufficient volumes of reaction mixtures shall be prepared to enable at least 10 PCRs to be carried out. As a general rule pipetting of volumes less than 5μl should be avoided.

### Table B1 — Components used in a typical PCR reaction

<table>
<thead>
<tr>
<th>Component a</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution water</td>
<td>Diluent</td>
</tr>
<tr>
<td>PCR buffer solution</td>
<td>The composition varies greatly according to the supplier and various additives [bovine serum albumin, dimethyl sulfoxide (DMSO), surface active agents, etc.] appropriate for the activity or stability of the thermostable DNA polymerase used, can be added</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium in its divalent cationic form is an essential co-factor of DNA polymerase activity. It forms a complex that is soluble with the dNTP. Its final concentration is thus dependent upon concentrations of dNTP, primers, probe, and target DNA. It shall be optimized</td>
</tr>
</tbody>
</table>
| dNTP | Deoxyribonucleotide triphosphates used in synthesizing DNA by polymerase DNA: b
- dATP: 2'-deoxyadenosine 5'-triphosphate;
- dTTP: 2'-deoxythymidine 5'-triphosphate;
- dCTP: 2'-deoxycytidine 5'-triphosphate;
- dGTP: 2'-deoxyguanosine 5'-triphosphate |
| Primers | Oligonucleotides of determined size and sequence that determine the specific sequence to be amplified by PCR |
| Thermostable DNA polymerase | Enzyme or mix of enzymes used for in-vitro DNA polymerase reaction. **NOTE** Use of hot-start Taq DNA polymerase is possible to avoid false-positive results. |
| Probes | Oligonucleotides of determined size and sequence that hybridize on to a specific portion of the amplicon and which bear a fluorophore enabling the recognition of the fragment |

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a Depending on their source, some of these components may previously be mixed in the PCR buffer solution (ready-to-use PCR master mix products including the components, except primers and probe are available).  
b A dTTP + dUTP (2'-deoxyuridine 5'-triphosphate) mix and a uracil-DNA N-glycosylase (UNG) enzyme can be used. This system is not mandatory for methods using a real-time detection system not requiring opening of tubes after amplification. Any equivalent system able to specifically destroy the amplicons from previous PCR, in the reaction mix, can be used.

B7.5.3 Other reagents

B7.5.3.1 DNA co-precipitants
Used to improve precipitation yield during DNA extraction, shall contain no nuclease activity or sequence homologous to the target sequences of the PCR tests.

B7.5.3.2 TE buffer, pH 8.0

Tris(hydroxymethyl)aminomethane (C$_4$H$_{11}$NO$_3$)  tris 10 mmol/l
Ethylenediaminetetraacetic acid (C$_{10}$H$_{16}$N$_2$)  EDTA 1 mmol/l
DNase- and RNAse-free water

Dissolve the tris and EDTA in DNase- and RNAse-free water and adjust with HCl to pH 8.0. For a 10-fold diluted TE buffer, dilute the solution with DNase- and RNase-free water.

B7.6 Additional information

B7.6.1 Decontamination of equipment and premises

After accidental or non-accidental contamination, any recyclable equipment or material shall be treated by immersing in or soaking with, for example, a solution of bleach with 1.7 % volume fraction active chlorine or 1% volume fraction hydrochloric acid or detergent.

Ultraviolet radiation can also be used to decontaminate small equipment or materials, counter tops or even an entire room in addition to decontamination solutions.

B7.6.2 Treatment and elimination of waste

Toxic and infectious waste shall be stored, used, and eliminated according to local regulations. It is recommended that consumables contaminated by amplification products be discarded within a day of their use.

B8 Analytical procedure

B8.1 Concentration

Filter as large a volume of the sample as practicable (usually 1 L) to concentrate the bacteria. Record the volume (V) of sample filtered. This is required to calculate the results (see Section 9). The limit of detection, LD$_{meth}$ and limit of quantification, LQ$_{meth}$, are adversely affected by small sample volumes and increase proportionally.

B8.2 DNA extraction

B8.2.1 General

Extraction involves freeing the DNA by lysing the microorganisms, then (or at the same time) purifying the DNA while eliminating the other components as much as possible, particularly the PCR inhibitors.

B8.2.2 Protocols

After filtration, the DNA can be directly extracted on the filter or a further step of cell concentration such as by membrane or immune-magnetic beads can be performed. In the last case, DNA can be extracted from the cell concentrated solution. It is recommended that the whole concentrate be treated. To extract the DNA, several suitable methods can be
used such as physical (e.g. cycles of freezing and thawing), chemical (e.g. guanidine thiocyanate buffer) or biological (e.g. enzyme digestion).

Purification can take place after or during DNA extraction. This purification can be performed, for example, using chloroform and/or by fractional precipitation, with solvents such as ethanol, isopropanol, and/or adsorption on solid matrices (e.g. resin, silica, glass, membrane, magnetic beads).

The purified DNA shall be put back into suspension in a solution that guarantees the stability of the DNA and the quality of the PCR, e.g. a buffer containing a magnesium-chelating agent (EDTA) or proteins (bovine serum albumin).

PCR quantification of *Legionella* spp. and *L. pneumophila* genomic units shall be performed with the same DNA extract.

B8.2.3 Stability of DNA extracts

DNA extracts shall be stored at 5°C ± 3°C and analysed within 24 h of preparation. Any longer storage at this temperature requires validation.

DNA extracts can be frozen below −18°C for several months; these storage conditions shall also be validated.

B8.3 DNA amplification by PCR

B8.3.1 General

This involves amplification of a limited target sequence in the 5’-to-3’ direction on each of the DNA strands by two additional primers. During the development of the PCR test, the amplification parameters (number of cycles, hybridization temperature, etc.) and the reaction mix composition (dNTP, magnesium, primers, and buffer) will have been defined and optimized. Once these parameters have been established, the performance of the method shall be assessed according to ISO/TS 12869 [6] or equivalent.

The PCR amplification shall include controls described in Section 10 (negative and positive controls, PCR inhibition control, and reference material).

B8.3.2 Target sequences, primers and probes

B8.3.2.1 General

One or more sequences can be amplified to detect and differentiate the DNA from bacteria belonging to *Legionella* spp. and *L. pneumophila*.

Therefore, primers and probes can be designed and used for the amplification of *Legionella* spp. and *L. pneumophila* provided that their specificity is assessed.

For storage conditions for primers and probes follow the manufacturer’s instructions, however it is advisable to prepare aliquots of the stock solution. It would be advisable to prepare ten times dilutions of the stock solution as a working solution at the using concentration to prevent continuous thawing/freezing samples. Use TE buffer pH 7.4 or pH 8 to prepare the dilutions.

B8.3.3 Amplification mix preparation
Ideally, prepare the reaction mixtures immediately before use. If the reaction mix is stored, then its stability requires validation by performing a verification of the linearity of the calibration function after storage.

The reaction mix and the extracted DNA shall be mixed just before amplification. To prevent the consequences of accidental contamination, PCR amplifications can be performed from dUTP to activate a UNG (uracil-DNA N-glycosylase) which removes all traces of amplicon before any new amplification.

The composition of a qPCR mix varies depending on the used reagents or commercial kits used. If using separate reagents and not a pre-formulated commercial kit the laboratory should perform a full validation of the reagents. A less comprehensive validation can be performed by the laboratory if using a commercially available kit. Performance data from the manufacturer of the kit should be consulted.

B8.4 Quantitative detection

B8.4.1 General

This detection shall enable detection and quantification of specific amplicons for *Legionella* spp. and/or *L. pneumophila*.

Specificity of the quantitative PCR shall be guaranteed by appropriately validated primers and specific hybridization probe(s) (when a Taqman assay is used). Alternative approaches may use double stranded DNA binding dyes. To ensure the quality of the quantitative detection, it is necessary to use a) and b).

a) An external DNA standard range, i.e. *L. pneumophila*-calibrated DNA solutions, derived from the primary standard (see Section 10.2).

b) A PCR inhibition control, such as a calibrated solution of plasmid or oligonucleotide or *L. pneumophila* genomic unit, co-amplified with the DNA from the sample. This approach shall be used to reveal any inhibitor presence in the sample DNA extract (see Section 10.6).

It is preferable to amplify the external calibrated standards and the inhibition control using the same primers used to amplify the target sequences of the sample.

In approaches a) and b), quantification is performed by interpolation within the linear response range of the DNA quantification method. This concentration range shall be determined beforehand. The extracted DNA can, if necessary, be diluted to obtain a concentration situated within this linear response area.

The amplification shall be performed with a real-time PCR thermocycler with a sufficient number of cycles which should not be less than the Ct value of the DNA concentration representing the limit of detection increased by 5.

IMPORTANT — it is advisable, whenever possible, to carry out replicate tests using the same DNA extract. Repeating tests and obtaining a mean result improves accuracy.

B8.4.2. Protocol
B8.4.2.1 Introduction

Temperatures and times included in the thermocycler program are dependent on the primers, probe, polymerase and buffer used. Moreover, the programme shall be adapted according to thermocycler model and laboratory conditions and shall be validated.

The programme shall be set in such a way that, during the DNA replication, the fluorescence signals of the Legionella spp. and L. pneumophila specific probe and the PCR internal control specific probe are measured.

B8.4.2.2 General

The following approach can be used for detection and quantification of amplicons. Monitoring of the PCR is based on the measurement of a fluorescent signal due to hybridization of at least one fluorescent labelled probe internal to the amplicon.

A working calibration range (external) comprising at least four levels (for example, solutions at 25 GU, 250 GU, 2,500 GU, and 25,000 GU of L. pneumophila per reaction tube) is prepared using the working calibration solution (commercial solution or solution prepared in-house). The first point of the DNA range shall be equal to the quantification limit LQ_{qPCR}.

The working calibration solution shall be connected to the Legionella DNA primary standard (see Section 10.2). An expiration date for this solution shall be set for the planned storage conditions and verified by coupling to the primary standard.

At least once during each sample amplification series (same PCR reagent batches, same materials), this working calibration range shall be analysed under the same conditions as that used for the samples.

The stability of the calibration within a series and/or the re-use of a diluted range shall be verified by measuring the reference material upon each use of the thermocycler (See Section 10.3).

B8.4.2.3 Real-time quantification

Aside from the fact that real-time thermocyclers can detect amplification products on site, they are also particularly appropriate for quantitative PCR. The detection systems enable the limits associated with the plateau effect to be circumvented by directly measuring the quantity of amplicon synthesized during the exponential amplification phase. These processes involve extremely sensitive fluorescent emission quantification and detection systems. The principle currently used for calibration is based on quantification of specific amplicons using at least one internal fluorescent labelled probe. Quantification is based on the determination of the cycle threshold, Ct, proportional to the decimal logarithm of the number of genomic units initially present in the reaction mix.

Several mathematical methods for determining Ct can be used. In this case, the method used shall be described and its effect in terms of measurement precision shall be checked by compliance with a standard curve evaluation protocol.

B9 Calculations and expression of results

Express the results according to Table B2 in number of genomic units (GU) of Legionella spp. and/or L. pneumophila per litre of sample (taking into account the concentrated volume
of water sample). If a volume of sample less than a litre is examined then the results should be expressed per volume analysed.

EXAMPLE 1 12,312 GU/l of *Legionella* spp. is expressed as “12,000 GU/L of *Legionella* spp.”

EXAMPLE 2 723 GU/l of *L. pneumophila* is expressed as “720 GU/L of *L. pneumophila*”. Keep a record of the volume filtered so it is possible to consider the dilution factor and the proportion between GU/well and GU/L.

### Table B2 — Expression of results

<table>
<thead>
<tr>
<th>Number N GU/PCR well</th>
<th>DNA dilution</th>
<th>Reported result GU/L</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N&lt;1</td>
<td>1</td>
<td>( \frac{\text{LD}_{qPCR} F}{V} )</td>
<td>Legionella(^a) not detected</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>( \frac{\text{LO}_{qPCR} dF}{V} )</td>
<td>DNA dilution due to the presence of PCR inhibitors <em>Legionella</em>(^a) not detected</td>
</tr>
<tr>
<td>1&lt;N&lt;LQ(_qPCR)</td>
<td>1</td>
<td>( \frac{\text{LO}_{qPCR} F}{V} )</td>
<td><em>Legionella</em>(^a) detected below the limit of quantification</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>( \frac{\text{LO}_{qPCR} dF}{V} )</td>
<td>DNA dilution due to the presence of PCR inhibitors <em>Legionella</em>(^a) detected below the limit of quantification</td>
</tr>
<tr>
<td>N&gt;LQ(_qPCR)</td>
<td>1</td>
<td>( \frac{NF}{V} )</td>
<td><em>Legionella</em>(^a) quantitatively detected</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>( \frac{NdF}{V} )</td>
<td>DNA dilution due to the presence of PCR inhibitors <em>Legionella</em>(^a) quantitatively detected</td>
</tr>
<tr>
<td>N&gt;C</td>
<td>0</td>
<td>( \frac{CF}{V} )</td>
<td><em>Legionella</em>(^a) detected above the limit of quantification (^b)</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>( \frac{CdF}{V} )</td>
<td>DNA dilution due to the presence of PCR inhibitors <em>Legionella</em>(^a) detected above the limit of quantification (^b)</td>
</tr>
</tbody>
</table>

\(N\) average number of GU/PCR well
LD\(_qPCR\) limit of detection determined according to 10.5
LQ\(_qPCR\) limit of quantification determined according to 10.4
\(C\) upper value of the calibration range determined according to 10.3
\(F\) conversion factor (No. of genomic units per well to No. of genomic units per litre)
\(d\) DNA dilution factor
\(V\) volume, in litres, of sample filtered
\(^a\) According to the PCR system, specify *Legionella* spp. or *L. pneumophila*.
\(^b\) In this case, the quantification can be obtained after DNA dilution.

### B10 Quality controls

#### B10.1 General
Quality controls ensure trueness and precision of measurements carried out by a laboratory. The stated frequencies of the controls are the minimum frequencies required when routinely setting up these techniques. The accumulation of results can allow these frequencies to be modified.

B10.2 Connecting the calibration solution and the reference material to the primary standard

B10.2.1 Principle

The trueness of the real-time PCR measurement is ensured by three levels of standards:

a) a primary standard;
b) working calibration solutions used with each amplification series;
c) a reference material connected to the primary standard, used without dilution as an external quantitative quality control;

The working calibration solutions (whether or not supplied in a commercial kit) shall be connected to the primary standard at least once a year. Moreover, the manufacturer of the commercial kit or the designing laboratory shall perform this connection while implementing any change to the calibration solutions.

The reference material shall be connected to the primary standard and stored aliquoted under validated conditions ensuring its homogeneity and stability.

B10.2.2 Protocol

To perform the connection, the working calibration solution shall be calibrated with the primary standard as follows.

From the working calibration solution to be connected, prepare at least three independent ranges with four levels (minimum) by serial dilutions, covering the linear quantification range, in the solution used for analysing the PCR blank. Perform the same with the primary standard. The target levels for these two solutions shall be equivalent. These two series of three independent DNA ranges shall be analysed in the same PCR series.

B10.2.3 Data analysis

a) Verification of the equivalence of the slopes (PCR efficiency).

By linear regression, establish the calibration function using the values obtained for the primary standard calibration range (called the reference range). Verify that the slope $a$ lies between $-4.115$ and $-2.839$ corresponding to amplification efficiency with a value between 75 % and 125 %. Most commercially available PCR platforms will perform this analysis automatically.

By reverse calibration, recalculate the decimal logarithm genomic unit values with the $C_t$ values obtained, using the calibration function, for each level of the working calibration range. For each level, calculate the deviation between the expected value and the recalculated value. Calculate the absolute value of the difference of the deviations at the highest point and lowest point of the range.
If this value is greater than 0.15, the slopes and therefore the efficiencies are not equivalent. Connection is not possible. If this value is less than or equal to 0.15, the slopes and therefore the efficiencies are equivalent.

b) *Readjustment of the working calibration solution.*

If the slopes are equivalent, calculate the mean of the deviations. If the absolute value of this mean is greater than 0.15, make another precise calibration solution (zero bias) by dilution from the stock solution.

Otherwise, no correction is necessary. Connection has been achieved.

Proceed in the same way to evaluate the value of the reference material or use the reference material available from a national *Legionella* reference centre.

An example of connection of the working calibration solution to the primary standard is given in Table B3.

**Table B3 – Example of connection of the working calibration solution to the primary standard**

<table>
<thead>
<tr>
<th>Reference range</th>
<th>Obtained CTs (cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level tested log$_{10}$(GI)</td>
<td>33.33</td>
</tr>
<tr>
<td>log$_{10}$(25)</td>
<td>31.64</td>
</tr>
<tr>
<td>log$_{10}$(250)</td>
<td>27.92</td>
</tr>
<tr>
<td>log$_{10}$(2 500)</td>
<td>24.64</td>
</tr>
<tr>
<td>log$_{10}$(25 000)</td>
<td>39.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calibration solution</th>
<th>Obtained CTs (cycles)</th>
<th>Mean C per level</th>
<th>Quantity found per level</th>
<th>Calibration error per level</th>
</tr>
</thead>
<tbody>
<tr>
<td>log$_{10}$(25)</td>
<td>34.55</td>
<td>34.34</td>
<td>34.62</td>
<td>34.50</td>
</tr>
<tr>
<td>log$_{10}$(250)</td>
<td>31.07</td>
<td>30.92</td>
<td>30.80</td>
<td>30.93</td>
</tr>
<tr>
<td>log$_{10}$(2 500)</td>
<td>27.02</td>
<td>27.70</td>
<td>27.73</td>
<td>27.48</td>
</tr>
<tr>
<td>log$_{10}$(25 000)</td>
<td>24.23</td>
<td>24.49</td>
<td>24.52</td>
<td>24.42</td>
</tr>
</tbody>
</table>

| Mean calibration error | 0.05 |

a) *Verification of the equivalence of the slopes (PCR efficiency):*

$|\text{calibration error} [\log_{10}(25,000) - \log_{10}(25)]| = |0.03 - (-0.01)| = 0.04 \leq 0.15$

The slopes of the two ranges are equivalent; verification of the calibration can be performed.
b) Readjustment of the working calibration solution.

The mean calibration error is less than $0.15 \log_{10}$, no calibration correction is necessary for the calibration solution.

NOTE Connection of the calibration solution cannot be extrapolated below or above the range established with the primary standard solution.

B10.3 Monitoring of the performances

B10.3.1 Calibration performances

The following shall be monitored:

— the values of the slopes for calibration curves (control charts);
— the value of the reference material (quantified by reverse calibration).

The calculated value shall correspond to the reference value $\pm 2 \times 0.15 \log_{10}$ unit. This control, expressed as a decimal logarithm of genomic units, shall be monitored over time (control chart).

B10.3.2 Monitoring of the performances at the limit of quantification

For each calibration: deviation from the model at the first calibration range point ($LQ_{\text{PCR}}$) shall be monitored. The absolute value of the deviation shall be less than $2 \times 0.15$. Laboratories may tighten the limits set by increasing the number of range points.

B10.4 Positive and negative controls of the method

As a positive control, carry out an assessment of the recovery at least once a month. This quantitative positive control is used to monitor over time (using for example control charts) the recovery of the method, initially determined during the performance evaluation phase.

A negative control of the method is performed by following the complete procedure on a sample volume from 100 mL to 1 L of Legionella DNA-free sterile water. This control shall be carried out after each series of extractions. The negative control sample shall be the last sample following filtration of all the other samples in the series. The negative control of the method is used to monitor the working environment. For methods that have been fully validated by a third party, the manufacturer’s instructions shall be thoroughly followed for the interpretation of the positive or negative controls and PCR reagent blank.

B10.5 PCR Blank

For each run of sample PCR amplifications, prepare a reagent blank to verify that there is no DNA contamination during PCR. The negative control can be used for that purpose. Nevertheless, the preparation of a blank specifically for the PCR step can be used to detect contaminations at this stage. This should avoid unnecessary investigation of the entire method if results are positive. A positive blank indicates contamination and requires special validation of the test. A PCR blank with a $C_t$ value greater than the validated limit of detection shall be considered negative.
B10.6 Inhibition control

B10.6.1 General

It is essential that the presence of PCR inhibitors in the DNA extract be assessed. An inhibition control shall be added to the sample extract. This inhibition control is either the target itself (see Section 10.6.2), or a plasmid or an oligonucleotide (see Section 10.6.3).

B10.6.2 The inhibition control is the target

Test at least one well with the extract from the sample (Well 1), one well with the PCR inhibition control alone (Well 2) and one well with the sample extract and the PCR inhibition control (Well 3).

The PCR curve of the inhibition control is the curve obtained with the reaction mix into which a known quantity of target DNA has been added.

The test of the presence of inhibitors consists of comparing the curve of the spiked sample extract (Well 3) with that of the control (Well 2). If the slopes are not parallel, there is possible inhibition. Dilute the sample DNA extract to confirm inhibition.

If the slopes in the exponential phase of the curves (i.e. the slopes of the tangents to the Ct) are parallel, then perform interpretation according to Table B4.

Table B4 – Interpretation of the inhibition control when the control is the target

<table>
<thead>
<tr>
<th>Sample extract (1)</th>
<th>Control (2)</th>
<th>Sample extract + control (3) compared with control (2)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_t_{1,1}$</td>
<td>$C_t_{1,2}$</td>
<td>$C_t_{1,3} \leq C_t_{1,2}$</td>
<td>Presence of <em>Legionella</em> spp. or <em>L. pneumophila</em> DNA</td>
</tr>
<tr>
<td>$C_t_{1,1}$</td>
<td>$C_t_{1,2}$</td>
<td>$C_t_{1,3} &gt; C_t_{1,2}$</td>
<td>Inhibition, sample DNA extract to be diluted until coherent $C_t$ values are obtained with added dose</td>
</tr>
<tr>
<td>No amplification</td>
<td>$C_t_{1,2}$</td>
<td>$C_t_{1,3} = C_t_{1,2}$</td>
<td>No <em>Legionella</em> spp. or <em>L. pneumophila</em> DNA at the detection threshold of the method</td>
</tr>
<tr>
<td>No amplification</td>
<td>$C_t_{1,2}$</td>
<td>$C_t_{1,3} &gt; C_t_{1,2}$</td>
<td>Inhibition, DNA extract from sample to be diluted until coherent $C_t$ values are obtained with added dose</td>
</tr>
</tbody>
</table>

B10.6.3 The inhibition control is either a plasmid or an oligonucleotide

The inhibition control is either a plasmid or an oligonucleotide possessing sequences complementary to primers used to amplify the *Legionella* spp. or *L. pneumophila* target. It is thus co-amplified with the target. Table B5 provides the qualitative interpretation of the inhibition control results. Analysis of diluted DNA is required if DNA purification is not correct depending on inhibition.
Table B5 – Interpretation of the inhibition control when this control is a plasmid or an oligonucleotide

<table>
<thead>
<tr>
<th>Multiplex amplification</th>
<th>Specific Legionella spp. or L. pneumophila sequence</th>
<th>Inhibition control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Complying</td>
<td>Legionella spp. or L. pneumophila DNA present</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>Not complying&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Partial inhibition or competition; the sample DNA extract shall be diluted until a positive inhibition control is obtained.</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>Complying</td>
<td>No Legionella spp. or L. pneumophila DNA at the detection limit of the method</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>Not complying&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Inhibition DNA extract from sample to be diluted until a positive internal control is obtained.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The inhibition control (IC) is not compliant if its C<sub>T</sub> value is significantly different from those observed on the sample inhibition control amplified within the nearest point of the calibration range. A drift of C<sub>T</sub> is considered as significant if the C<sub>T</sub> value of the IC does not fall into the μ<sub>C</sub><sub>T</sub><sub>±3σ<sub>C</sub>T</sub> interval (where μ<sub>C</sub>T and σ<sub>C</sub>T are, respectively, the average and the standard deviation of C<sub>T</sub> values of internal inhibition controls of the different calibration range solutions).

For a third party fully validated method, manufacturer's instructions shall be thoroughly followed.

D11 Test report

This test report shall contain at least the following information:

a) the test method used,
b) all the information required to identify and describe the sample
c) sampling date and conditions
d) the filtered volume of the sample
e) the results expressed as described in Section 9.
f) any details not included in this Technical Specification that may have an effect on the results

B12 References


The determination of Legionella bacteria in water and other environmental samples (2019) - Part 3 - Method for their detection and quantification by polymerase chain reaction (qPCR) and protocol for method validation.


**Address for correspondence**

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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