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Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)

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National foreword

This Published Document is the UK implementation of ISO/TS 12869:2019. It supersedes PD ISO/TS 12869:2012, which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/3/4, Microbiological methods.

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Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)

Qualité de l'eau — Détection et quantification de Legionella *spp. et/ou* Legionella pneumophila *par concentration et amplification génique par réaction de polymérisation en chaîne quantitative (qPCR)*

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This second edition cancels and replaces the first edition (ISO/TS 12869:2012), which has been technically revised. The main changes compared to the previous edition are as follows:

— meet expectations from customers and governments faced with *Legionella* risk;

— information on management, especially needing a fast result, has been updated;

— the use of new technologies while overseeing the development work of various actors in the sector has been allowed;

— the return of experiences from the laboratories using this method since 2006 has been taken into account;

— in Annex G, information on evolution of the requirements for the use of third party validated commercial kits has been added.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The presence of *L. pneumophila* or *Legionella* spp. in water samples is demonstrated and quantified by amplifying DNA sequences (PCR) with specific oligonucleotides. Specificity of the detection is ensured by using a target sequence specific fluorescent-labelled probe. The increase in the amount of the DNA amplicon can be measured and visualized in real time by a quantitative PCR device with fluorophore specific filters.

A calibration curve is used for quantification purposes. The guidelines, minimum requirements and performance characteristics are intended to guarantee that the results are reliable and reproducible between different laboratories.

This document specifies a determination of the recovery of the DNA extraction. The performance of the extraction procedure is not fully covered (lysis efficiency is not estimated).

Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)

WARNING — *Legionella* spp. shall be handled safely by experienced microbiologists on the open bench in a conventional microbiology laboratory conforming to containment level 2. Infection by *Legionella* spp. is caused by inhalation of the organism; hence it is advisable to assess all techniques for their ability to produce aerosols. In case of doubt, carry out the work in a safety cabinet.

1 Scope

This document specifies a method for the detection and quantification of *Legionella* spp. and *L. pneumophila* using a quantitative polymerase chain reaction (qPCR). It specifies general methodological requirements, performance evaluation requirements, and quality control requirements.

Technical details specified in this document are given for information only. Any other technical solutions complying with the performance requirements are suitable.

NOTE 1 For performance requirements, see Clause 9.

This document is intended to be applied in the bacteriological investigation of all types of water (hot or cold water, cooling tower water, etc.), unless the nature and/or content of suspended matter and/or accompanying flora interfere with the determination. This interference can result in an adverse effect on both the detection limit and the quantification limit.

NOTE 2 For validation requirements, see 9.7.

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

The method described in this document is applicable to all types of water. However, some additives, such as chemicals used for water treatment, can interfere with and/or affect the sensitivity of the method.

The qPCR methods do not give any information about the physiological state of the *Legionella.*

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms, definitions, symbols and abbreviated terms

3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1.1
Legionella
<genotype definition> bacterial genus which can be defined by DNA sequences of genes encoding its specific 16S rRNA

Note 1 to entry: rRNA is the abbreviation of ribosomal ribonucleic acid.

3.1.2
Legionella pneumophila
<genotype definition> species belonging to the *Legionella* (3.1.1) genus which can be defined by its specific DNA sequences

Note 1 to entry: The distinction between *Legionella* spp. and *L. pneumophila* can be made on the basis of the difference between the nucleotide sequence in the macrophage infectivity potentiator (*mip*) gene.

3.1.3
reverse primer
forward primer
single-strand DNA fragment (oligonucleotide) that serves as a template for specific DNA replication

Note 1 to entry: The choice of the DNA sequences of both the forward and reverse primers determines which DNA fragment is replicated. The length of the primer usually varies from 15 to 30 nucleotides.

3.1.4
probe
single-stranded DNA fragment, targeting a specific sequence, labelled with a fluorophore reporter and a fluorophore quencher

Note 1 to entry: While the probe is unattached or attached to the template DNA and before the polymerase acts, the quencher reduces the fluorescence from the reporter.

3.1.5
quantitative PCR
qPCR
formation of specific DNA fragments which is highlighted by a labelled fluorescent probe and monitored in real time

Note 1 to entry: The intensity of the fluorescence is a measure of the amount of amplicons. By comparison with a calibration curve, the initial concentration of the DNA target can be determined.

3.1.6
C_t **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 1 to entry: The C_t value is the intercept of the line that represents the DNA concentration of a sample with fluorescent base line. C_t value is equivalent to Cq value depending on the software used.

3.1.7
***Legionella* spp. genome unit**
GU
unit representing a single copy of the *Legionella* spp. bacterial genomic DNA

3.1.8
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 1 to entry: 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*.

3.1.9
PCR inhibition control
calibrated DNA that is required to be co-amplified with the sample DNA extract using the primers needed for *Legionella* spp. or *L. pneumophila* detection

Note 1 to entry: The PCR inhibition control should reveal any inhibitor presence in the sample DNA extract.

Note 2 to entry: The control can be a plasmid, an oligonucleotide or the *L. pneumophila* genomic DNA. A specific probe shall be used to detect the inhibition control.

3.1.10
recovery
efficiency of the DNA extraction method

3.1.11
***Legionella pneumophila* DNA primary standard**
calibrated DNA solution of *L. pneumophila* (WDCM 00107) with a known quantity of genome units and an associated uncertainty

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

Note 2 to entry: For the WDCM catalogue, see Reference [3].

3.1.12
reference material
ready-to-use calibrated DNA solution connected to the *L. pneumophila DNA primary standard* (3.1.13)

Note 1 to entry: The reference material shall be processed in each PCR run to check the accuracy of the qPCR.

3.1.13
amplification series
set of PCR amplification runs while using the same PCR reagent batches, same materials, and same instruments

3.1.14
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 1 to entry: The procedure is specified in 7.4.

3.1.15
Taq DNA polymerase
enzyme from *Thermophilus aquaticus* used for in vitro DNA polymerase reaction

3.1.16
negative control
control for monitoring the whole process in this method (from filtration to extraction to qPCR)

3.1.17
MgCl2
magnesium in its divalent cationic form is an essential co-factor of DNA polymerase activity

Note 1 to entry: It forms a complex that is soluble with the dNTP.

3.1.18
dNTP
deoxyribonucleotide triphosphates used in synthesizing DNA by polymerase DNA:

— dATP: 2'-deoxyadenosine 5'-triphosphate;

— dTTP: 2'-deoxythymidine 5'-triphosphate;

— dCTP: 2'-deoxycytidine 5'-triphosphate;

— dGTP: 2'-deoxyguanosine 5'-triphosphate

3.2 Symbols and abbreviated terms

LD_{qPCR}	(detection limit of the qPCR) lowest number of genome units that give a positive result in the qPCR with 90 % confidence
LD_{meth}	(detection limit of the qPCR) lowest number of genome units that might be detected in the volume of sample filtrated
LQ_{qPCR}	(quantification limit of the qPCR) lowest number of genome units that can be quantified with an accuracy less than or equal to $0{,}15\log_{10}$unit
LQ_{meth}	(quantification limit of the qPCR) lowest number of genome units that might be quantified in the volume of sample filtrated
BSA	bovine serum albumine
DMSO	dimethyl sulfoxide

4 Principle

The detection and quantification of *Legionella* spp. or *L. pneumophila* by PCR are carried out in three phases:

— concentration of water samples by filtration;

— DNA extraction from the filter;

— amplification, detection and quantification of one or more specific DNA sequences belonging to the *Legionella* genus and/or *L. pneumophila* species by real-time qPCR.

5 Sampling

The samples shall be taken in sterile containers using all the necessary precautions. The sampling conditions shall be indicated on the test report if they are known. Carry out sampling, transport and storage of the samples in accordance with ISO 19458. Take care not to expose the samples to adverse temperature conditions (e.g. freezing or overheating).

NOTE The use of insulated containers is helpful in this regard.

Preferably, start the investigation after the sampling as soon as possible. If samples are delivered to the laboratory 24 h after sampling, they can be shipped at (5 ± 3) °C or at ambient temperature (20 ± 5) °C. In case the conservation period is more than 24 h, the shipment shall be performed at (5 ± 3) °C.

Validate the storage of the filter membrane or the sample for a longer time or at another temperature.

In addition, for samples derived from oxidizing biocide-treated water a sterile container, which contains a sufficient quantity of sterile sodium thiosulfate, shall be used for neutralizing the oxidizer.

Other biocides (bactericides or bacteriostatics) are sometimes used, in particular in cooling tower circuits. Their presence, which can lead to underestimation, shall thus be declared and indicated on the test report if it is known. However, it is not always possible to neutralize these products.

6 General testing conditions

6.1 General

PCR is a sensitive detection method. Aerosols, dust, and other particles are carriers of contaminating DNA. It is therefore essential to separate in space and/or time the different stages of the analysis. In particular, provide separate dedicated areas, materials, and equipment for pre- and post-amplification stages.

The principles to be applied are as follows:

— use of disposables compatible with PCR methods is preferred;

— procedures for eliminating DNA traces and amplicons shall be implemented in event of accidental contamination of the premises or apparatus;

— regular quality controls shall be used to demonstrate the effectiveness of maintenance procedures with the objective of ensuring that there is no contaminating *Legionella* DNA or PCR products/amplicons (see 10.4).

6.2 Staff

All personnel who perform this method shall be trained for working with PCR and microbiological aspects.

The staff shall wear separate laboratory coats for microbiology activities involving cultures and molecular biology activities. Any gloves that are used for this purpose shall be talc-free.

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

More information about this subject is available in the “Quality Assurance/Quality Control Guidance for Laboratories Performing PCR analyses on Environmental Samples” from EPA (see Reference [4]).

6.3 Premises

The laboratory shall contain at least two physically separated areas (e.g. PCR cabinet), the area including pre-PCR [a) and b) below] and PCR [c) below] activities. Ideally, there should be three physically separated areas a), b), and c) available:

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 PCR amplification.

If automated machines are used, then certain activities can be grouped together in the same area. In all cases, check on contaminations by using a negative control (see 10.4).

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

6.4 Apparatus and consumables (excluding reagents)

6.4.1 Apparatus

Usual laboratory equipment, and in particular the following.

6.4.1.1 Biological safety cabinet (BSC II).

6.4.1.2 Centrifuge.

6.4.1.3 Heating block module.

6.4.1.4 Real-time thermocycler.

Device used for amplification by PCR which, after each cycle of polymerization, detects and records a fluorescent signal which is proportional to the amount of amplification product (genome units).

6.4.2 Consumables

All used consumables shall be free of DNA and DNAse.

EXAMPLE Filter funnels can be:

— delivered sterile;

— sterilized in an autoclave or oven;

— if made of metal, flamed prior to use.

6.4.3 Concentration

Membrane filters shall be made of polycarbonate 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 membrane filters containing cellulose or glass fibre.

6.4.4 Extraction and PCR (detection and quantification)

6.4.4.1 General

Apart from the concentration phase, it is important to avoid the apparatus coming into contact with the water sample to prevent cross-contamination. Avoid cross-contamination by using single-use disposables.

The quality control shall be used to confirm the effectiveness of the decontamination protocols. Wherever possible, use consumables which are suitable for molecular diagnostics.

Careful consideration should be given to the apparatus and consumables specified in 6.4.1 and 6.4.2.

6.4.3.2 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.

6.4.3.3 Heating blocks, recommended, to prevent contamination by aerosols.

6.4.3.4 BSC II, ideally equipped with UV lamps to ensure decontamination of equipment used.

6.5 Reagents

6.5.1 General

All reagents used shall be sterile, free from nucleases and PCR inhibitors. Ideally, they should be 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.

Perform initial non-contamination control of the batch of reagents which are used for the DNA isolation and qPCR (as described in 10.4).

6.5.2 PCR reagents

An example of a PCR reaction mix components is indicated in Table 1. Ready-to-use PCR master mix products including the different components, except primers and probe, are available.

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.

Table 1 — Example of a typical PCR reaction mix

Component[a]	Comments
Dilution water	Diluent
PCR buffer solution	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.
MgCl2	The final concentration MgCl2 depends on the dNTP, primers, probe, and target DNA concentrations. This shall be optimized:
dNTP	— dATP: 2'-deoxyadenosine 5'-triphosphate;
	— dTTP: 2'-deoxythymidine 5'-triphosphate;
	— dCTP: 2'-deoxycytidine 5'-triphosphate;
	— dGTP: 2'-deoxyguanosine 5'-triphosphate.
	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.
Primers	See 7.3.2.2, 7.3.2.3, 7.3.2.5, 7.3.2.6.
Thermostable DNA polymerase	Use of hot-start Taq DNA polymerase is possible to avoid false-positive results.
Probes	See 7.3.2.4 and 7.3.2.7.
[a] Depending on their source, some of these components may previously be mixed in the PCR buffer solution.	

6.5.3 Other reagents

6.5.3.1 DNA co-precipitants, used to improve precipitation yield during DNA extraction, shall not contain any nuclease activity or sequence homologous to the target sequences of the PCR tests.

6.5.3.2 TE buffer, pH 8,0.

Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$)	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.

6.6 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.

6.7 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 immediately.

7 Procedure

7.1 Concentration

Filter as large a volume of the sample as practicable (at least 50 ml) to concentrate the bacteria. Record the volume (V) of sample filtered. This is required to calculate the results (see Clause 8). The limit of detection, LD_{meth} (see 9.5) and limit of quantification, LQ_{meth} (see 9.4.4), are adversely affected by small sample volumes and increase proportionally.

7.2 DNA extraction

7.2.1 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. Check the recovery of the extract DNA (see 9.6).

7.2.2 Protocols

The DNA can be directly extracted from the filter. It is recommended to process the whole concentrate.

To extract the DNA, several suitable methods can be used such as physical (e.g. cycles of freezing and thawing, beads beating), chemical (e.g. guanidine thiocyanate buffer) or biological (e.g. enzyme digestion).

Purification step can be performed after or simultaneous of the DNA extraction step. This purification step 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, for example, a buffer containing a magnesium-chelating agent (EDTA) or proteins (bovine serum albumin).

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

7.2.3 Stability of DNA extracts

After the DNA extraction, the DNA extract can be used for PCR. Although it is recommended to perform the PCR directly after the extraction it is possible to store the DNA extract for 24 h at (5 ± 3) °C. Any longer storage at this temperature requires validation.

In case the DNA extract is stored for longer than 24 h, store the DNA extract at (-18 ± 2) °C; these storage conditions shall be validated.

7.3 DNA amplification by PCR

7.3.1 General

This involves amplification of a limited target sequence in the 5'-to-3' direction on each of the DNA strands initiated by two primers (reverse primer and forward primer).

During the development of the PCR test, the amplification parameters (number of cycles, hybridization temperature) and the reaction mix composition (dNTP, magnesium, primers, and buffer) shall be defined and optimized. Once these parameters have been established, the performance of the method shall be evaluated (see Clause 9).

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

7.3.2 Target sequences, primers and probes

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

The specificity of the primers and probes shall be checked:

a) theoretically by homology research using appropriate software in the main databases such as NCBI Genbank (see Reference [1]) or EMBL Nucleotide sequence database (see Reference [2]);

b) by testing on strains of *Legionella*, *L. pneumophila* and strains of microorganisms likely to be found in the same ecological niches as *Legionella*.

Regarding b), a list of the minimum number of strains to be tested is given in 9.2. For strains not belonging to the genus *Legionella*, no amplification product shall be detected by the real-time PCR. The specificity of the probes and primers shall be evaluated on each new strain of legionella For *L. pneumophila* the sequences described below are compatible with the list of strains to be tested for specificity. Other sequences may be used as long as they match the exclusivity and inclusivity requirements (see the list in 9.2).

There follow examples of primers (7.3.2.2 and 7.3.2.3) and probes (7.3.2.4) designed to amplify and quantify the *L. pneumophila* specific fragment of *mip* (7.3.2.5). Sequences and fluorofors are given for exemple.

These preparations are given as examples and shall be validated according to Clause 9.

7.3.2.2 Example of Forward primer *L. pneumophila*: LpneuF, with the following composition.

Sequence: 5'-CCGATGCCACATCATTAGC-3'[6];

TE buffer (6.5.3.2): diluted 10 times.

LpneuF is prepared, for example, as follows. Prepare a stock solution of primers in 10 times diluted TE buffer at a final concentration of 100 µmol/l. Store this stock solution below –18 °C. Dilute the stock solution to a working solution of 10 µmol/l. For the preparation of both the stock solution and the working solution, use a 10 times diluted TE buffer. Store this working solution for up to six months below –18 °C.

7.3.2.3 Example of Reverse primer *L. pneumophila*: LpneuR, with the following composition.

Sequence: 5'-CCAATTGAGCGCCACTCATAG-3'[6];

TE buffer (6.5.3.2): diluted 10 times.

NOTE LpneuR is prepared, for example, as in a similar fashion to LpneuF (7.3.2.2).

7.3.2.4 Example of Probe *L. pneumophila*: LpneuP, with the following composition.

Sequence: 5'-TGCCTTTAGCCATTGCTTCCG-3'[6];

Label 5': Fluorophore (carboxyfluorescein, FAM);

Label 3': Quencher (black hole quencher 1, BHQ1);

TE buffer (6.5.3.2).

NOTE LpneuP is prepared, for example, as in a similar fashion to LpneuF (7.3.2.2).

7.3.2.5 Example of Forward primer 16S rRNA gene forward primer *Legionella* spp.: 16SrRNAgeneF, with the following composition.

Sequence: 5' GGAGGGTTGATAGGTTAAGAGCT 3'[5];

TE buffer (6.5.3.2): diluted 10 times.

16SrRNAgeneF is prepared, for example, as follows. Prepare a stock solution of primers in 10 times diluted TE buffer at a final concentration of 100 µmol/l. Store this stock solution below –18 °C. Dilute the stock solution to a working solution of 10 µmol/l. For the preparation of both the stock solution and the working solution, use a 10 times diluted TE buffer. Store this working solution for up to six months below –18 °C.

7.3.2.6 Example of Reverse primer 16S rRNA gene reverse primer *Legionella* spp.: 16SrRNAgeneR, with the following composition.

Sequence 5': CCAACAGCTAGTTGACATCGTTT 3'[5];

TE buffer (6.5.3.2): diluted 10 times.

NOTE 16SrRNAgeneR is prepared in a similar fashion to LpneuF (7.3.2.5).

7.3.2.7 Example of Probe *Legionella* spp. 16S rRNA geneP, with the following composition.

Sequence: 5’ -AGTGGCGAAGGCGGCTACCT- 3’[5];

Label 5’: Fluorophore (carboxyfluorescein, FAM);

Label 3’: Quencher (black hole quencher 1, BHQ1);

TE buffer (6.5.3.2).

NOTE 16SrRNAgeneP is prepared in a similar fashion to LpneuF (7.3.2.5).

7.3.2.8 DNA sequence of *mip* fragment of *L. pneumophila*.

Primers and probes sequences are identified in bold in the text (Reference [5]).

5'**CCGATGCCACATCATTAGC**TACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCCGATTTGGGGAAGA
ATTTTAAAAATCAAGGCATAGATGTTAATC**CGGAAGCAATGGCTAAAGGCA**TGCAAGACG**CTATGAGTG
GCGCTCAATTGG**-3'

7.3.2.9 DNA sequence of 16S rRNA gene fragment of *L. species*.

Primers and probes sequences are identified in bold in the text (see Reference [5]).

TGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTGAGGGTTGTAAAGCACT
TTCAGTGGGGAGG**AGGGTTGATAGGTTAAGAGC**TGATTAACTGGACGTTACCCACAGAAGAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGGG
TGCGTAGGTGGTTGATTAAGTTATCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTT
GACTCGAGTATGGGAGAGGGTAGTGGAATTTCCGGTGTAGCGGTGAAATGCGTAGAGATCGGAAGGAACA
CCAGTGGCGAAGGCGGCTACCTGGCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCTGTAAA**CGATGTCAACTAGCTGTTGG**TTATATGAAAATAATTAGTGGCG
CAGCAAACGCGATAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCTTGACATACA
GTGAATTTTGCAGAGATGCATTAGTGCCTTCGGGAACACTGATACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTATCCTTAGTTGCCAGCATGTGATGGT
GGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAGTCATCATGGCCCTT
ACGGGTAGGGCTACACACGTGCTACAATGGCCGATACAGAGGGCGGCGAAGGGGCGACCTGGAGCAAATCC
TTAAAAGTCGGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG.

7.3.3 Amplification mix preparation

Ideally, prepare the reaction mix 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 (see 9.3). This validation shall meet the criteria defined in 9.3.4.

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.

An example of a composition of a reaction mix is given in Table 2. Mix the components in the proportion indicated in Table 2.

7.4 Quantitative detection

7.4.1 General

This real-time PCR based method shall enable quantification of specific amplicons for *Legionella* spp. and/or *L. pneumophila*. It is recommended to perform the qPCR for *Legionella* spp. and *L. pneumophila* in two separate PCR wells. This document does not describe a multiplex detection. A multiplex detection (*Legionella* spp. and *L. pneumophila* in the same PCR well) is not applicable to the content of this document.

The specificity of the quantitative PCR shall be ensured by using specific hybridization probe(s).

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 10.2).

b) A PCR inhibition control, such as a calibrated solution of plasmid or oligonucleotide or *L. pneumophila* genome 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 10.6). The amplification of the PCR inhibition control will be detected using a specific probe designed with a different reporter than the probe of the target.

Regarding the detection (presence or absence) of the target, a result shall be considered as a positive (presence of the target) when the C_t value is lower than the C_t value corresponding to 1 GU. This C_t value is estimated (b) during the characterization and the validation of the method (see 9.3.2) in the linear equation $y = ax' + b$.

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

The quantity of the PCR inhibition control shall be lower than 1 000 cDNA per reaction.

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 during the characterization and the validation of the method (9.3). 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. This number of cycles shall not be less than the estimated value of the ordinate intercept (refer to 9.3) increased by 5.

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

Table 2 — Example of composition of the qPCR mix

Reagents	Volume per sample Ml	Final concentration
BSA for PCR applications	5	0,4 µg/µl
Taq polymerase[a]	1	0,1 U/µl
PCR buffer (Table 1)	5	1 ×
dNTPs (Table 1)	1	400 nmol/l
$MgCl_2$[a] (Table 1)	3	According to the Taq DNA polymerase requirements
Primer LpneuF (7.3.2.2)	1	200 nmol/l
PCR inhibition control shall be added according to 9.6.		
[a] The volume required depends on the concentration in the stock solutions and can vary with supplier.		

Table 2 *(continued)*

Reagents	Volume per sample Ml	Final concentration
Primer LpneuR (7.3.2.3)	1	200 nmol/l
Probe LpneuP (7.3.2.4)	1	200 nmol/l
Water for PCR applications	make up to 40	
PCR inhibition control shall be added according to 9.6. [a] The volume required depends on the concentration in the stock solutions and can vary with supplier.		

7.4.2 PCR protocol

7.4.2.1 Overview

The thermocycler program in Table 3 is indicated as an example for the detection of *L. pneumophila* by using the primers and probe sequences specified in 7.3.2.

This program shall be adapted according to the model and the type of the thermocycler and shall be validated according to the requirements stated in Clause 9.

Table 3 — Example of temperature and time program of PCR

Denaturing of DNA and activation of hot-start Taq polymerase	3 min at 95 °C
DNA replication	20 s at 95 °C 60 s at 60 °C
Number of cycles	43[a]
[a] In this table, this number of cycle is determined as the intercept of the calibration function + 3 in order to be able to observe the amplification profil obtained for 1 GU.	

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

NOTE The duration of the hot-start step depends on the Taq DNA polymerase which is used for the qPCR and is stated on the product specification from the manufacturer.

7.4.2.2 General

The following approach can be used for detection or 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 according to Annex A). 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 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 reuse of a diluted range shall be verified by measuring the reference material upon each use of the thermocycler (see 10.3).

7.4.2.3 Real-time quantification

Aside from the fact that real-time thermocyclers can detect amplification products in situ, 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 the cycle threshold, C_t, inversely proportional to the decimal logarithm of the number of genome units initially present in the reaction mix.

A method for determining the C_t is given as an example in Annex B.

Other mathematical methods for determining C_t 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 the standard curve evaluation protocol (see 9.3).

7.5 Qualitative detection

In case of qualitative detection, the use of an external calibration is not necessary.

The C_t limit value which shall be taken into account to determine the presence of the target (*Legionella pneumophila* or *Legionella* spp.) can be determined if standards curves with intercept values are available, an average intercept value (corresponding to Ct 1 GU defined in 7.4) can be used, above which results can be considered as negative.

A PCR result with a C_t value later than the C_t value of the intercept b (see 9.3.4.1) shall be considered as negative.

The qualitative interpretation may be acceptable for methods otherwise validated as quantitative (by the laboratory itself or the supplier).

8 Expression of the results

Express the results according to Table 4 in number of genome units (GU) of *Legionella* spp. and/or *L. pneumophila* per litre of sample (taking into account the filtered volume of water sample) to two significant figures.

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. pneumophil*a is expressed as “720 GU/l of *L. pneumophil*a”.

For the expression of the results for qualitative detection, see Table 5.

Table 4 — Expression of results for quantitative detection

Number N GU/PCR well	**DNA dilution**	**Reported result** GU/l	**Comment**
$N < 1$	1	$< \frac{LD_{qPCR} F}{V}$	*Legionella*[a] not detected
	d	$< \frac{LD_{qPCR} dF}{V}$	DNA dilution due to the presence of PCR inhibitors *Legionella*[a] not detected
$1 < N < LQ_{qPCR}$	1	$< \frac{LQ_{qPCR} F}{V}$	*Legionella*[a] detected below the limit of quantification
	d	$< \frac{LQ_{qPCR} dF}{V}$	DNA dilution due to the presence of PCR inhibitors *Legionella*[a] detected below the limit of quantification
$N > LQ_{qPCR}$	1	$\frac{NF}{V}$	*Legionella*[a] quantitatively detected
	d	$\frac{NdF}{V}$	DNA dilution due to the presence of PCR inhibitors *Legionella*[a] quantitatively detected
$N > C$	0	$> \frac{CF}{V}$	*Legionella*[a] detected above the limit of quantification[b]
	d	$> \frac{CdF}{V}$	DNA dilution due to the presence of PCR inhibitors *Legionella*[a] detected above the limit of quantification[b]

where

N is the average number of GU/PCR well;

LD_{qPCR} is the limit of detection determined according to 9.5;

LQ_{qPCR} is the limit of quantification determined according to 9.4;

C is the upper value of the calibration range determined according to 9.3;

F is the conversion factor (No. of genome units per well to No. of genome units per test portion);

d is the DNA dilution factor;

V is the volume, in litres, of sample filtered.

[a] According to the target, specify *Legionella* spp. or *L. pneumophila.*

[b] In this case, the quantification can be obtained after DNA dilution.

Table 5 — Expression of results for qualitative detection

Number *N* GU/PCR well	**DNA dilution**	**Reported result** GU/l	**Comment**
$N < 1$	1	$< \frac{\mathrm{LD}_{\mathrm{qPCR}} F}{V}$	*Legionella*[a] not detected
	d	$< \frac{\mathrm{LD}_{\mathrm{qPCR}} dF}{V}$	DNA dilution due to the presence of PCR inhibitors *Legionella*[a] not detected
$N >= 1$	1	*Legionella*[a] detected	
	d	*Legionella*[a] detected	

where

N is the average number of GU/PCR well;

$\mathrm{LD}_{\mathrm{qPCR}}$ is the limit of detection determined according to 9.5;

F is the conversion factor (No. of genome units per well to No. of genome units per test portion);

d is the DNA dilution factor;

V is the 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.

9 Technical protocol for the characterization and the validation of the method

9.1 General

The technical criteria and requirements described in this clause shall be used for the characterization 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 Clause 9.

For third party validated commercial methods that fulfil the requirements given in Clause 9, the manufacturer's instructions shall be accurately followed.

For primary validation, all requirements stated in Clause 9 are applicable. For method verification, (secondary validation), simplified requirements shall be used for the validation of the correct implementation in the laboratory of any third party fully validated method, as specified in Annex G.

Laboratories using only qualitative detection shall fulfil the requirements described in Clause 9 except 9.3 and 9.4

9.2 Inclusivity and exclusivity of probes and primers

Primers and probes used shall provide the expected results for the following species and serogroups that have all been isolated in environment or from human clinical case.

Inclusivity and exclusivity tests shall be performed on all species listed below. DNA extracts should be obtained in part from WDCM and/or national collection strains and in part from environmental

strains. All the environmental strains analysed as part of this validation shall be documented including the alternative method used to identify the strain as *Legionella*.

— Inclusivity tests are carried out on DNA extracts containing about 100 GU/PCR well. This spiking dose avoids false-positive result.

— Exclusivity tests are carried out on DNA extracts containing at least 10 000 GU/well. This spiking dose avoids false-negative result.

Bacteria suspension concentrations can be estimated using the optical density of the suspension at 600 nm. (An optical density of 0,5 at 600 nm corresponds to 10^9 CFU/ml).

— Inclusivity list (microorganisms to test for a *Legionella* spp. method): *L. anisa* (e.g. WDCM 00106), , *L. birminghamensis, L. bozemanii 1 and 2, L. cherrii, L. cincinnatiensis* (e.g. CIP 103875) , *L. dumoffii, L. erythra 2, L. feeleii 1 and 2, L. gormanii, L. hackeliae* serogroup1 (e.g. CIP 103844) *and serogroup 2, L. jordanis, L. lansingensis* (e.g. ATCC 43751), *L. longbeachae 1 and 2, L. maceachernii, L. micdadei* (e.g ATCC 33218), *L. oackridgensis, L. parisiensis* (e.g. CIP 103847), *L. pneumophila* serogroups *1 to 15* (e.g. *Legionella pneumophila* serogroup 1 WDCM 00107; *Legionella pneumophila* serogroup 6 ATCC 33215), *L. sainthelensi 1 and 2, L. tucsonensis, L. wadsworthii* (e.g. CIP 103886).

— Inclusivity list (microorganisms to test for a *L. pneumophila* method): 15 serogroups from the species.

— Exclusivity list (tested microorganisms recognized as not belonging to *Legionella* genus and/or being phylogenetically close). At least the following list shall be tested: *Aeromonas hydrophila, Alcaligenes faecalis* (e.g. CIP 60.80), *Bacillus subtilis* (e.g. CCM 1999), *Burkholderia cepacia, Clostridium spp., Enterobacter aerogenes, Escherichia coli* (e.g. WDCM 00003), *Flavobacterium spp., Klebsiella oxytoca* (e.g. WDCM 00179), *Listeria monocytogenes* (e.g. WDCM 00109 or WDCM 00020), *Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Serratia marcescens, Stenotrophomonas maltophila*.

— Exclusivity list (tested microorganisms recognized as not belonging to *L. pneumophila* species): *L. anisa* (e.g. WDCM 00106), *L. bozemanii, L. dunmofii, L. gormanii, L. jordanis, L. longbeachae, L. micdade* (e.g. ATCC 33218)*i, L. parisiensis* (e.g. CIP 103847), *L. tucsonensis*.

All the strains analysed as part of this validation shall be documented including the alternative method used to identify the strain as *Legionella*.

9.3 Verification of the calibration function of the quantitative PCR phase

9.3.1 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.

9.3.2 Calibration curve verification principle

Experience has shown that the means of C_t 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 formula such as $y = ax' + b$.

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

The parameters of the calibration curve are initially determined by following the evaluation protocol described in 9.3.3. A statistical analysis (see 9.3.4) is then performed in order to:

a) determine the formula for the line (see 9.3.4.1);

b) verify the linear regression model (see 9.3.4.3).

9.3.3 Calibration curve evaluation protocol

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

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, 2 500, 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 9.4). 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 6.

Perform the calculations as indicated in Table 6.

Table 6 — Formatting of results and calculations

Level x_i	x_1 = LQ_{PCR}	x_2 = 10 LQ_{PCR}	x_3 = 100 LQ_{PCR}	x_4 = 1 000 LQ_{PCR}	x_p	**Totals**
$x_i^{'} = \log_{10} x_i$	$x_1^{'}$	$x_2^{'}$	$x_3^{'}$	$x_4^{'}$	$x_p^{'}$	
$y_{i,j}$ (k repetitions)	$y_{1,1}$	$y_{2,1}$	$y_{3,1}$	$y_{4,1}$	$y_{p,1}$	
	$y_{1,2}$	$y_{2,2}$	$y_{3,2}$	$y_{4,2}$	$y_{p,2}$	
	$y_{1,k}$	$y_{2,k}$	$y_{3,k}$	$y_{4,k}$	$y_{p,k}$	
$T_i = \sum_{j=1}^{k} y_{i,j}$	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	
$x_i^{'} T_i$	$x_1^{'} T_1$	$x_2^{'} T_2$	$x_3^{'} T_3$	$x_4^{'} T_4$	$x_p^{'} T_p$.	$\sum_{i=1}^{p} x_i^{'} T_i$

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

$x_i^{'}$ is the logarithm of x_i;

$y_{i,j}$ is the C_t value measurement at level i (i = 1 … p) and row j (j = 1 … k);

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

p is the number of levels ($P \geq 4$).

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

$$N = k_p \tag{1}$$

See Annex C for a full calculation example.

9.3.4 Analysis of the results

9.3.4.1 Estimation of the regression curve

The regression curve is given by Formula (2):

$$y = \mu_{C_t} = ax' + b \tag{2}$$

where μ_{C_t} is the average C_t 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 Formulae (3) and (4):

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

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

Proceed to the following calculations in order to determine the slope a. The variance of x'_i, V_{x_i} is given by Formula (5):

$$V_{x'_i} = \frac{\Sigma x'^2_i - \left[\left(\Sigma x'_i\right)^2\right] / N}{N-1} \tag{5}$$

and the covariance of $x'y$, $\sigma_{x'y}$, by Formula (6):

$$\sigma_{x'y} = \frac{\Sigma x'_i T_i \left(\sum_i x'_i T_G / N\right)}{N-1} \tag{6}$$

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

$$a = \frac{\sigma_{x'y}}{V_{x'_i}} \tag{7}$$

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

$$\overline{x'} = \frac{\sum x'}{N}$$

and on the ordinate

$$\overline{y} = \frac{T_G}{N}$$

Consequently,

$$\overline{y} = \overline{ax'} + b$$

and therefore

$$b = \overline{y} - \overline{ax'} = \frac{T_G}{N} - a\frac{\sum x'}{N}$$

9.3.4.2 Estimate and verification of the efficiency

Efficiency assesses the yield of the PCR reaction.

Efficiency, e, is calculated using Formula (8):

$$e = \left(10^{-1/a} - 1\right) \times 100 \tag{8}$$

Efficiency shall have a value between 75 % and 125 %. Consequently, the value of the slope, a, shall be between −4,115 and −2,839. This range of acceptable slopes is large enough to include all qPCR systems on all thermocyclers. For a defined qPCR system on one thermocycler used in routine, the limit of acceptance should be estimated as any quality control using a statistical study or quality chart.

If a is outside the range, the amplification system shall not be validated.

9.3.4.3 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 shown in Formula (9):

$$E_{\text{lin}} \leq 0{,}15 \tag{9}$$

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

To do this, proceed to the calculations given in Table 7.

If $E_{\text{lin}i} \leq 0{,}15$, 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 $0{,}15 \log_{10}$unit, the model shall not be validated.

NOTE Examination of the bias values and standard deviations shows whether the model error is caused by a precision issue (s'_i too high) or a bias issue ($\overline{x'}_i - x'_i$ .too high).

Table 7 — Bias, precision, accuracy and uncertainty of linearity calculations

Estimated x_i level	x_1	x_2	x_3	x_4	x_p
Theoretical x'_i	x'_1	x'_2	x'_3	x'_4	x'_p
$x'_{i,j}$	$x'_{1,1}$	$x'_{2,1}$	$x'_{3,1}$	$x'_{4,1}$	$x'_{p,1}$
	$x'_{1,2}$	$x'_{2,2}$	$x'_{3,2}$	$x'_{4,2}$	$x'_{p,2}$
	$x'_{1,3}$	$x'_{2,3}$	$x'_{3,3}$	$x'_{4,3}$	$x'_{p,3}$
	$x'_{1,4}$	$x'_{2,4}$	$x'_{3,4}$	$x'_{4,4}$	$x'_{p,4}$
	$x'_{1,k}$	$x'_{2,k}$	$x'_{3,k}$	$x'_{4,k}$	$x'_{p,k}$
$\overline{x'_i} = \frac{\sum x'_{i,j}}{k}$	$\overline{x'_1}$	$\overline{x'_2}$	$\overline{x'_3}$	$\overline{x'_4}$	$\overline{x'_p}$
Bias, $\overline{x'_i} - x'_i$	$\overline{x'_1} - x'_1$	$\overline{x'_2} - x'_2$	$\overline{x'_3} - x'_3$	$\overline{x'_4} - x'_4$	$\overline{x'_5} - x'_5$
$s'_i = \sqrt{\frac{\sum_{j=1}^{k} x'^{2}_{i,j} - \left[\left(\sum_{k=1}^{k} x'_{i,j}\right)^2 / k\right]}{k-1}}$	s'_1	s'_2	s'_3	s'_4	s'_p
$E_{\text{lin}i} = \sqrt{s'^{2}_i + \left(\overline{x'_i} - x'_i\right)^2}$	$E_{\text{lin}1}$	$E_{\text{lin}2}$	$E_{\text{lin}3}$	$E_{\text{lin}4}$	$E_{\text{lin}p}$
$U_{\text{lin}i} = E_{\text{lin}i} t_{k-2}$	$U_{\text{lin}1}$	$U_{\text{lin}2}$	$U_{\text{lin}3}$	$U_{\text{lin}4}$	$U_{\text{lin}p}$

Theoretically

x'_i is the value calculated using the following formula: $x'_i = \log x'_i$;

$x'_{i,j}$ is the value calculated using the standard curve from the measurement value $y_{i,j}$;

$\overline{x'_i}$ is the average of $x'_{i,j}$;

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

E_{lin} is the accuracy of linearity;

U_{lin} is the expanded uncertainty of linearity;

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

9.3.5 Use of the calibration curve

For each measurement $y = C_t$ of a sample, use the standard curve formula, as shown in Formula (10), to obtain x' by inverse calibration:

$$x' = \frac{y - b}{a} \tag{10}$$

where k values of x' are obtained if k separate measurements of the same sample are taken.

Calculate the average of x' ($\overline{x'}$) and the associated standard deviations, according to the formula specified in Table 7.

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):

$$x = 10^{\overline{x'}} \tag{11}$$

9.4 Verification of the PCR limit of quantification, LQ_{qPCR}

9.4.1 Principle

The lowest acceptable limit of quantification is 25 GU ($1{,}40\log_{10}$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 $0{,}15\log_{10}$unit.

NOTE The $0{,}15\log_{10}$unit value comes from experimental data.

9.4.2 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 10.2). 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.

9.4.3 Analysis of results

Calculate the standard deviation for the x'_i values obtained via inverse calibration from the k measurements as shown in Formula (12):

$$s = \sqrt{\frac{\sum_{j=1}^{k} x_i'^2 - \left[\left(\sum_{k=1}^{k} x_i'^2\right)^2 \Big/ k\right]}{k-1}} \tag{12}$$

where

x'_i is the decimal logarithm of the number of genome units of *L. pneumophila*, calculated by inverse calibration, from the C_t values and the calibration curve formula;

k is the number of measurements.

Calculate the bias using Formula (13):

$$\overline{x'_i} - \log_{10}(x) \tag{13}$$

where x is the theoretical value of the targeted LQ_{qPCR}.

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

$$E_{LQ} = \sqrt{s^2 + \left[\overline{x'_i} - \log_{10(x)}\right]^2} \tag{14}$$

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):

$$U_{LQ} = E_{LQ} t_{tab} \tag{15}$$

where

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

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

An example of LQ_{qPCR} verification for a targeted LQ_{PCR} at 25 GU, with 10 measurements, is given in Table 8.

Table 8 — Example of LQ_{qPCR} verification

Test No.	x'_i
1	1,498
2	1,577
3	1,461
4	1,48
5	1,515
6	1,531
7	1,442
8	1,422
9	1,547
10	1,499
$\overline{x'_i}$	1,497
Bias	0,099
S	0,048
E_{LQ}	0,110
U_{LQ}	0,249

In the example in Table 8 E_{LQ} = 0,11, therefore $E_{LQ} \leq 0{,}15$, and the limit of quantification at 25 GU is validated.

9.4.4 Theoretical limit of quantification of the whole method

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

$$LQ_{meth} = \frac{LQ_{PCR} F}{V} \tag{16}$$

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_{meth} does not take into account the recovery inherent to the preparatory phases.

9.5 Verification of the PCR limit of detection (LDqPCR)

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_{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_{qPCR} from 10 separate dilutions prepared from a DNA solution of *L. pneumophila* connected to the primary standard (see 10.2).

9.6 Recovery method

9.6.1 Principle

The recovery study shall be carried out on DNAse/RNAse free water samples (without *Legionella* DNA) that have been artificially contaminated with dilutions of a mother suspension formed from a strain of *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.

9.6.2 Protocol

To perform a series of tests, create a mother suspension from colonies of *L. pneumophila* (WDCM 00107), then aseptically inoculate the *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.

Another way to make suspensions of *Legionella pneumophila* bacteria for recovery experiments is to use commercially available reference materials with a certified amount (expressed in GU) of *Legionella pneumophila*.

Calculate the average value, *A*, expressed in decimal logarithm of 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.

9.6.3 Calculations

The recovery calculation for a sample is obtained by Formula (17):

$$\log_{10}\eta_x = B - A + D + \log_{10}\frac{1\,000}{V_{pe}} \tag{17}$$

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;

B is 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.

9.7 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 perform this, estimate for each type of matrix the recovery (e.g. cooling tower water, potable water, surface water, waste water) and follow over time (optional control charts). Follow the protocol described in 9.6, 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 9.6.

9.8 Measurement uncertainty of the whole method

The uncertainty measurement of the whole method encompasses both accuracy and intermediate precision.

The approach described in this document is based on the analysis of the recovery values.

The bias is estimated by the average recovery value for all the matrices (9.6 and 9.7). The precision is estimated through the recovery variance, using all the values obtained during the initial validation of the method (9.6), the robustness study (9.7), and the monitoring of recovery over time (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 9 (see example in Table F.1, Annex F).

Table 9 — Calculation of uncertainty from recovery values

Sample No.	Matrix	Level tested	Sample recovery
$x = 1 \ldots n$ where n is the total number of samples for all matrices and all levels together	Sterile water	Level 1 (e.g. 1 000 GU/l) Level 2	η_x
	Hot sanitary water	Level 1 Level 2	
	Air cooling water	Level 1 Level 2	
	etc.	Level 1 Level 2	
Average recovery $\left(\bar{\eta}_x\right)$			$\dfrac{\sum_{x=1}^{n}\eta_x}{n}$
Variance (s^2)			$s^2 = \dfrac{\sum_{x=1}^{n}\eta_x^2 - \left[\left(\sum_{x=1}^{n}\eta_x\right)^2 \Big/ n\right]}{n-1}$
Overall expanded uncertainty, U_{overall}			$U_{\text{overall}} = 2\times\sqrt{\eta_x^{-2} + s^2}$

Another estimation of the uncertainty is possible with an interlaboratory study; see Annex H.

10 Quality controls

10.1 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.

In case of qualitative detection, all the quality controls have to be performed except those described in 10.2 and 10.3.

10.2 Connecting the calibration solution and the reference material to the primary standard

10.2.1 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 (see 10.3).

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. A reference material [*L. pneumophila* (WDCM 00107) DNA solution] connected to a primary standard, which shall be used without dilution, is available from the French national legionella reference centre.

10.2.2 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.

10.2.3 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 % (see 10.3).

   By reverse calibration, recalculate the decimal logarithm genome 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 0,20 the slopes and therefore the efficiencies are not equivalent. Connection is not possible.

   If this value is less than or equal to 0,20 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 0,20 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 10.

Table 10 — Example of connection of the working calibration solution to the primary standard

Reference range			
Level tested Log_{10}(GU)	**Obtained C_ts (cycles)**		
Log_{10}(25)	33,33	34,90	**34,68**
Log_{10}(250)	31,64	31,05	**31,18**
Log_{10}(2 500)	27,92	27,99	**27,80**
Log_{10}(25 000)	24,64	24,71	**24,60**
Slope	**−3,31**		
Intercept point	**39,10**		
Calibration solution			

Estimated level Log_{10}(GU)	**Obtained C_ts (cycles)**			**Mean C_t per level**	**Quantity found per level**	**Calibration error per level**
Log_{10}(25)	34,55	34,34	34,62	34,50	1,39	**−0,01**
Log_{10}(250)	31,07	30,92	30,80	30,93	2,47	**0,07**
Log_{10}(2 500)	27,02	27,70	27,73	27,48	3,51	**0,11**
Log_{10}(25 000)	24,23	24,49	24,52	24,42	4,43	**0,03**
Mean calibration error						**0,05**

c) Verification of the equivalence of the slopes (PCR efficiency):

$$|\text{calibration error } [\log_{10}(25\,000) - \log_{10}(25)]| = |0{,}03 - (-0{,}01)| = 0{,}04 \leq 0{,}20$$

The slopes of the two ranges are equivalent; verification of the calibration can be performed.

d) Readjustment of the working calibration solution.

The mean calibration error is less than $0{,}20\log_{10}$, 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.

10.3 Monitoring of the performances

10.3.1 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 ±0,4 $\log_{10}$unit.

This control, expressed as a decimal logarithm of genome units, shall be monitored over time (control chart).

10.3.2 Monitoring of the performances at the limit of quantification

For each calibration: deviation from the model at the first calibration range point (LQ_{PCR}) shall be monitored. The absolute value of the deviation shall be less than 2 × 0,15. Laboratories may tighten the limits set by increasing the number of range points.

10.4 Positive and negative controls of the method

As a positive control, carry out an assessment of the recovery at least once a month according to 9.6. 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 (see 9.6).

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 filtrations. 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 whole process (from filtration to extraction to qPCR).

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 NTC (see 10.5).

In case of qualitative detection, the positive control is a sample spiked with a *Legionella pneumophil*a suspension without any requirement of quantification.

10.5 No template control (NTC)

For each run of sample PCR amplifications, prepare a NTC to verify that there is no DNA contamination during PCR.

The negative control (10.4) 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 NTC with a C_t value greater than the C_t value of the intercept point shall not be considered as positive.

10.6 Inhibition control

10.6.1 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 10.6.2), or a plasmid or an oligonucleotide (see 10.6.3).

10.6.2 The inhibition control is the target

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

The PCR curve of the inhibition control is the curve obtained with the reaction mix (6.5.2) into which a known quantity of target DNA has been added.

The test of the presence of inhibitors consists in comparing the curve of the spiked sample extract[3] with that of the control[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 C_t) are parallel, then perform interpretation according to Table 11.

Table 11 — Interpretation of the inhibition control when the control is the target

Sample extract (1)	Control (2)	Sample extract + control (3) compared with control (2)	Interpretation
$C_{t,1}$	$C_{t,2}$	$C_{t,3} \leq C_{t,2}$	Presence of *Legionella* spp. or *L. pneumophila* DNA
$C_{t,1}$	$C_{t,2}$	$C_{t,3} > C_{t,2}$	Inhibition, sample DNA extract to be diluted until coherent C_t values are obtained with added dose
No amplification	$C_{t,2}$	$C_{t,3} = C_{t,2}$	No *Legionella* spp. or *L. pneumophila* DNA at the detection threshold of the method
No amplification	$C_{t,2}$	$C_{t,3} > C_{t,2}$	Inhibition, DNA extract from sample to be diluted until coherent C_t values are obtained with added dose

$C_{t,1}$ Cycle threshold in the well with sample extract only.

$C_{t,2}$ Cycle threshold in the well with the control only.

$C_{t,3}$ Cycle threshold in the well with both sample extract and the control.

10.6.3 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 coamplified with the target. Table 12 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 12 — Interpretation of the inhibition control when this control is a plasmid or an oligonucleotide

Multiplex amplification		Interpretation
Specific *Legionella* spp. or *L. pneumophila* sequence	**Inhibition control**	
+	Complying	*Legionella* spp. or *L. pneumophila* DNA present
+	Not complying[a]	*Legionella* spp. or *L. pneumophila* DNA present Partial inhibition or competition, the sample DNA extract shall be diluted until a positive inhibition control is obtained.
–	Complying	No *Legionella* spp. or *L. pneumophila* DNA at the detection limit of the method
–	Not complying[a]	Inhibition, DNA extract from sample to be diluted until a positive internal control is obtained.

[a] The inhibition control (IC) is not compliant if its C_t value or its slope is significantly different from those observed on the sample inhibition control amplified within the nearest point of the calibration range. A drift of C_t is considered as significant if the C_t value of the IC does not fall into the $\mu_{c_t} \pm 3s_{c_t}$ interval (where μ_{c_t} and s_{c_t} are, respectively, the average and the standard deviation of C_t 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.

11 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO/TS 12869:2019;

b) all the information required to identify and describe the sample;

c) sampling date and conditions;

d) the analysis date;

e) the filtered volume of the sample;

f) the results expressed as described in Clause 8;

g) a treatment after 24 h of sampling ou any details not included in this document that may have an effect on the results.

Annex A
(informative)

Example of protocol for producing a quantitative standard DNA solution

A culture is prepared in a liquid medium (BCYE) at 37 °C ± 2 °C from a colony of *L. pneumophila* (WDCM 00107) isolated on the selective medium.

The optical density is measured at 600 nm. The exponential growth phase is obtained for an optical density value of 0,5 ± 0,1.

The DNA is extracted according to the laboratory's protocol.

After purification of the DNA and treatment with RNase (to degrade the residual RNA), the quantity and quality of the *L. pneumophila* DNA is measured. These are obtained by reading the optical density at 260 nm and 280 nm.

The ratio of the optical density obtained at 260 nm to the optical density obtained at 280 nm should be between 1,7 and 2,0 to show the quality of the extracted DNA. An optical density ratio less than 1,7 shows unsatisfactory purity of the extracted DNA and above 2,0 indicates the quantity of DNA is overestimated due to traces of RNA: the RNase action was not complete.

Also, it is possible to check the quality and quantity of DNA by an electrophoretic migration in gel with a semiquantitative marker.

The concentration of DNA solution (purified), in micrograms per litre, is obtained by Formula (A.1):

$$[\mathrm{DNA}] = \frac{A(260)}{20} \tag{A.1}$$

where

[DNA] is the concentration of DNA solution (purified) expressed in milligrams per millilitre;

$A(260)$ is the optical density measured at 260 nm.

The DNA extract is diluted to obtain the highest point in the range (e.g. 160 000 GU/ 5 µl, i.e. a DNA concentration of 688 000 fg/5 µl).

10^{-1} dilutions down to the LQ value are prepared.

DNA solutions is stored at –20 °C.

Annex B
(informative)

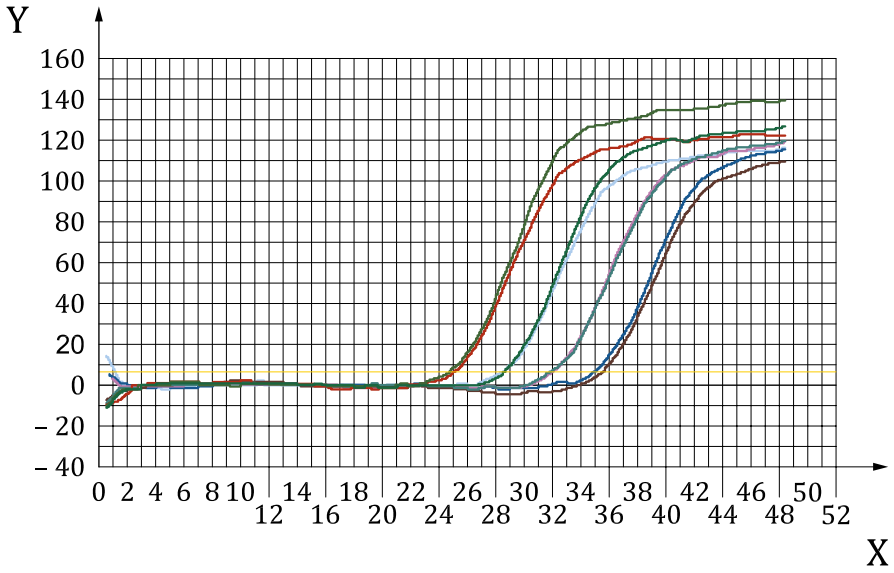
Example of method for determining the cycle threshold

Real-time PCR monitoring is carried out by reading fluorescent emission at each cycle. This signal is directly proportional to the number of genome units present in the reaction well. Background noise is exceeded after a number of cycles (corresponding to the C_t) which depends upon the initial number of genome units.

The quantification of unknown samples is obtained by using the C_t of the sample and the calibration function.

In the following example, the range points are 30 GU, 300 GU, 3 000 GU, and 30 000 GU in the PCR wells.

For example, at the end of PCR, the profiles below can be obtained (see Figure B.1).



Key

X cycle

Y PCR baseline subtracted RFU

NOTE In this example the slope is –3,26 and the intercept 39,1.

Figure B.1 — Profiles obtained

The data are reprocessed using the calibration range. To do this, determine the range of cycles during which the fluorescence measurement does not exceed the background noise (in this example, the first 22 cycles) and the position of the threshold on the fluorescence scale (in this example, at value 6) at the bottom of the exponential cycle and above the background noise.

C_t corresponds to the value of the abscissa of the intersection point of the fluorescence curve and the threshold. This corresponds to a number of cycles.

The function

$$C_t = f(\log_{10} n_{iGU})$$

where niGU is the initial number of genome units, can be determined from the Ct of the range points.

Formula (B.1) is obtained by linear regression (see also 9.3.4.1):

$$y = ax' + b \tag{B.1}$$

where

y is the C_t value;

a is the slope of the calibration curve from which PCR efficiency e is calculated;

b is the intercept point (theoretical C_t corresponding to one genome unit, $C_{t,1\ GU}$);

x' is $\log_{10} n_{iGU}$ where n_{iGU} ($n_{iGU} = 10^{[x']}$) is the initial number of genome units:

$$x' = (y - b)/a \tag{B.2}$$

Annex C
(informative)

Example of a study of the quantitative PCR phase calibration function

C.1 Table of data and calculations

The calculations presented in this example were rounded off to facilitate the presentation of the data. In practice, the calculations shall be carried out without rounding off the values.

See Table C.1.

Table C.1 — Data and calculations

Level x_i	**30**	**300**	**3 000**	**30 000**	**Sums**
$x'_i = \log_{10} x_i$	1,48	2,48	3,48	4,48	
$y_{i,j}$, k = 5 repetitions	35,18	31,07	27,27	23,97	
	34,84	31,41	27,58	24,12	
	34,80	31,15	27,36	24,06	
	34,48	31,21	27,52	24,21	
	34,80	31,42	27,55	24,11	
$T_i = \sum_{j=1}^{k} y_{i,j}$	174,10	156,26	137,28	120,47	T_G = 588,11
$m_i = \frac{T_i}{k}$	34,82	31,25	27,46	24,09	
$x'_i T_i$	257,17	387,52	477,34	539,36	$\sum_{i=1}^{p} x'_i T_i = 1\,662{,}63$
where x_i is the number of genome units of *Legionella* spp. or *L. pneumophila* per reaction tube; x'_i is the decimal logarithm of x_i; $y_{i,j}$ is the C_t value measurement at level i (i = 1 … p) and row j (j = 1 … k); k is the number of repetitions per level i, k = 5; p is the number of levels, P = 4.					

$N = kp = 5 \times 4 = 20$ according to Formula (1).

C.2 Estimation of the regression curve

C.2.1 Calculation of required elements

$$\sum_{i=1}^{p} x'_i = l\left(x'_1 + x'_2 + x'_3 + x'_4 + \ldots + x'_p\right) = 5\times 1,48 + 2,48 + 3,48 + 4,48) = 59,6$$

according to Formula (3)

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

$$5\times\left(1,48^2 + 2,48^2 + 3,48^2 + 4,48^2\right) = 202,608$$

according to Formula (4).

C.2.2 Calculation of the slope *a*

The variance of x'_i is given by

$$V_{x'_i} = \frac{\Sigma {x'_i}^2 - \left[\left(\Sigma x'_i\right)^2 / N\right]}{N-1} = \frac{202,608 - \left(59,6^2 / 20\right)}{19} = 1,316$$

according to Formula (5). The covariance of $x'y$ is given by

$$\sigma_{x'y} = \frac{\Sigma x'_i T_i - \left(\sum_i x'_i T_G / N\right)}{N-1} = \frac{1\,662,63 - \left(59,6\times 588,11 / 20\right)}{19} = -4,733$$

according to Formula (6). Hence, the slope is

$$a = \frac{\sigma_{x'y}}{V_{x'_i}} = -3,597$$

according to Formula (7) “a” is between −4,155 and −2,839 and amplification system is verified.

C.2.3 Calculation of intercept point *b*

The curve passes through the mean point, whose abscissa is

$$\bar{x}' = \frac{\sum x'}{N} = \frac{59,6}{20} = 2,98$$

and whose ordinate is

$$\bar{y} = \frac{T_G}{N} = \frac{588,11}{20} = 29,4055$$

hence

$$\bar{y} = \overline{ax'} + b$$

and thus

$$b = \bar{y} - \overline{ax'} = \frac{T_G}{N} - a\frac{\sum x'}{N} = 29,4055 - \left(-3,597 \times 2,98\right) = 40,12$$

The regression equation is as follows according to Formula (2):

$$y = \mu_{c_t} = ax' + b = -3,597x' + 40,12$$

C.3 Verification of efficiency

Efficiency *e* is given by Formula (8):

$$e = \left(10^{-1/a} - 1\right) \times 100 = 89,66\,\%$$

where *e* is between 75 % and 125 % and efficiency is verified.

C.4 Estimation of linearity performance

The performance of the linearity is estimated using the calculations indicated in Table 6. The values obtained are shown in Table C.2.

Table C.2 — Bias, precision, accuracy and uncertainty of linearity calculations

x_i **estimated level**	**30**	**300**	**3 000**	**30 000**
Theoretical x'_i	1,477 1	2,477 1	3,477 1	4,477 1
$x'_{i,j}$	1,396 7	2,523 6	3,565 5	4,470 4
	1,489 9	2,430 4	3,480 5	4,429 2
	1,500 9	2,501 7	3,540 9	4,445 7
	1,588 6	2,485 2	3,4970	4,404 6
	1,500 9	2,427 6	3,488 8	4,432 0
$\sum_{j=1}^{k} x_{i,j}$	7,477 0	12,368 5	17,572 7	22,181 8
$\overline{x'_i} = \frac{\sum x'_{i,j}}{k}$	1,495 4	2,473 7	3,514 5	4,436 3
Bias, $\overline{x'_i} - x'_i$	0,018 3	−0,003 4	0,037 4	−0,040 7
$s'_i = \sqrt{\frac{\sum_{j=1}^{k} x'^{2}_{i,j} - \left[\left(\sum_{j=1}^{k} x'_{i,j}\right)^2 / k\right]}{k-1}}$	0,068 0	0,043 0	0,036 8	0,024 1
$E_{\text{lin}i} = \sqrt{s'^{2}_i + \left(\overline{x'_i} - x'_i\right)^2}$	0,070 4	0,043 2	0,052 5	0,047 3
$U_{\text{lin}i} = E_{\text{lin}i} t_{k-2}$	0,224 1	0,137 3	0,167 0	0,150 5
$x_i = 10^{\overline{x'_i}}$	31	298	3 270	27 312

As $E_{\text{lin}i} \leq 0{,}15$, for all levels, the linearity is thus verified for the whole range.

Annex D
(informative)

Specific Student distribution

Table D.1 shows a Student distribution for a level of risk of α = 5 % for a bilateral test (1 – α/2).

Table D.1 — Specific Student distribution

α	0,05
1 – α/2	0,975
1	12,706
2	4,303
3	3,182
4	2,776
5	2,571
6	2,447
7	2,365
8	2,306
9	2,262
10	2,228
11	2,201
12	2,179
13	2,160
14	2,145
15	2,131
16	2,120
17	2,110
18	2,101
19	2,093
20	2,086
25	2,060
30	2,042
40	2,021
50	2,009
60	2,000
100	1,984
∞	1,960

Annex E
(informative)

Example of recovery evaluation

Pick up to 5 colonies from 72H *Legionella* culture and inoculate into 9 ml tryptone salt tube.

Take a 250 µl sample of stock suspension and mix with 1,25 ml of lysis buffer (final volume: 1,5 ml). The lysate is then diluted to 10^{-2} and 5 µl of this dilution is analysed by PCR. Repeat three times to obtain three lysates.

The PCR measurements performed on the three direct lyses give an average of 8 300 GU/5 µl.

The concentration in $\log_{10}$GU/ml of stock suspension is

$$A = \log_{10}\left(\frac{8\,300}{5}\times 100\times 1\,500\times\frac{1\,000}{250}\right) = 9$$

To produce spiked solutions, 250 µl of dilutions d_3 (10^{-3}) or d_5 (10^{-5}) of stock suspension are inoculated. For example, a PCR quantification of 1 580 GU inoculated is obtained for the 100 000 GU level.

$B = \log_{10}(1\,580) = 3{,}2$

$$\log_{10}\eta_x = 3{,}2 - 9 + 5 + \log_{10}\left(\frac{1\,000}{250}\right) = -0{,}2$$

according to Formula (17).

See Table E.1.

Table E.1 — Example of the mean recovery table obtained on two levels

Level	Sample recovery	Average recovery	Standard deviation
100 000 GU	−0,24	−0,09	0,17
	−0,02		
	−0,22		
	−0,18		
	0,09		
	−0,10		
	−0,29		
	0,20		
	−0,25		
	0,07		
1 000 GU	−0,09	0,12	0,16
	0,18		
	0,33		
	0,08		
	0,37		
	−0,12		
	0,14		
	0,26		
	−0,19		
	0,26		

Annex F
(informative)

Example of overall uncertainty evaluation

Table F.1 — Example of overall uncertainty evaluation

Level	Sample recovery			Mean recovery	Variance
	Sterile water	Hot sanitary water	Cooling tower water		
100 000 GU	−0,24	−0,37	−0,30	−0,218	0,105
	−0,02	−0,68	0,14		
	−0,22	−0,77	−0,61		
	−0,18	−0,45	−0,81		
	0,09	−0,13	−0,07		
	−0,10	−0,59	−0,38		
	−0,29	−0,74	−0,40		
	0,20	−0,66	−0,38		
	−0,25	−0,58	−0,20		
	0,07	−0,67	−0,27		
1 000 GU	−0,09	−0,41	0,18		
	0,18	−0,42	0,55		
	0,33	−0,40	−0,61		
	0,08	−0,48	−0,64		
	0,37	0,27	−0,27		
	−0,12	−0,56	−0,46		
	0,14	−0,09	0,21		
	0,26	−0,20	−0,15		
	−0,19	−0,18	−0,47		
	0,26	−0,07	−0,24		

$$U_{\text{overall}} = 2 \times \sqrt{-0,218^2 + 0,105} = 0,78$$

Annex G
(normative)

Evaluation of the performances of a third party validated method

G.1 Principle

The protocol of the method verification described in the following corresponds to a secondary validation of the method, applicable for any implementation of a third party validated method in a laboratory.

This protocol can supersede the requirements of Clause 9 only if:

— the implanted method has been satisfactorily certified or validated beforehand by a third party (primary validation, according to the requirements given in Clause 9);

— the validated method is implemented in the laboratory in its entirety (same material, unchanged protocol);

— in case of use of the method for a qualitative detection, provider (manufacturer) of a third party validated method should or could provide an intercept value (b in the linear equation $y = ax + b$ as defined in 7.4) for a "presence/absence" use.

G.2 Verification criteria

The verification criteria includes the following:

— inclusivity/exclusivity: not applicable. The validation file shall be supplied by the manufacturer;

— evaluation of the calibration curve: at least five ranges under intermediate reproducibility conditions;

— verification of the LQ_{qPCR}: to verify on the first point LQ_{PCR} of five calibration ranges analysed under intermediate reproducibility conditions;

— verification of the LD_{qPCR}: to verify on a single target concentration (LD), at the rate of 10 replicates, on 2 d (intermediate reproducibility);

— recovery: (see robustness);

— robustness: to realize according to the requirements given in 9.6 on sterile water samples and a complex matrix encountered in the laboratory (e.g. cooling tower water supplied by surface water). The recovery study shall be carried out on routine matrices encountered in the laboratory on a total number of samples greater or equal to 8;

— in case of evolution of a commercial kit, already third party validated, only the parameters impacted by the modification shall be verified;

— in the context of this annex, the connecting of the calibration solution and the reference material to the primary standard is not to be verified.

Annex H
(informative)

Interlaboratory studies

In this annex, precision has been evaluated from data of 14 interlaboratory comparisons (ILCs) performed between 2008 and 2014 and organized by AGLAE (French association of laboratories).

Materials similar to hot sanitary waters were used. Samples were prepared using public water supply neutralized, then spiked with different bacteria strains. Several strains, including sometimes mixed culture, were used:

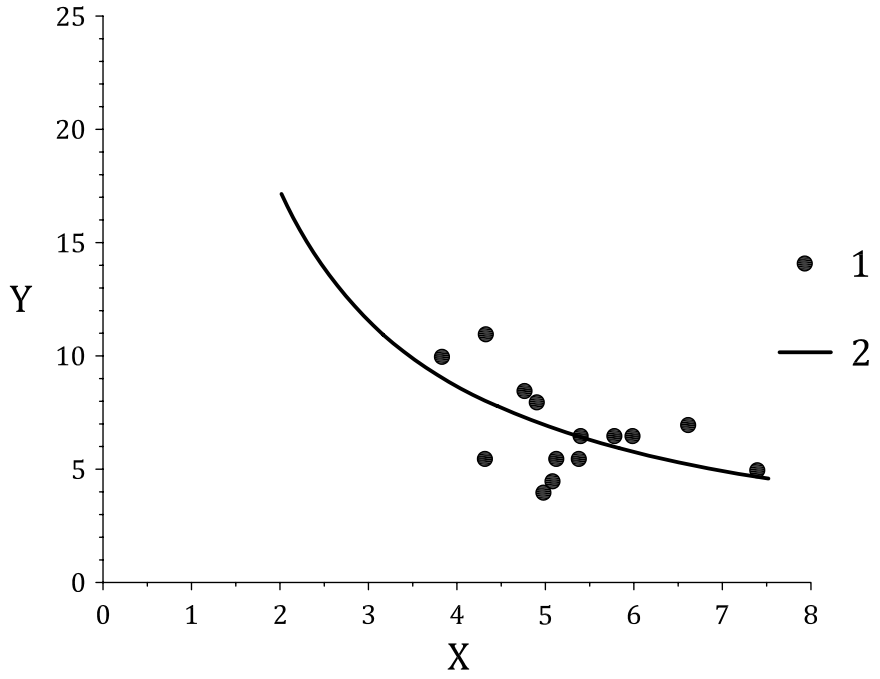
— *Legionella pneumophila serogroup 1, Legionella pneumophila serogroup 6*;

— *Legionella micdadei, Legionella bozemanii, Legionella dumoffi, Legionella anisa*;

— Background flora could be present in the samples.

The numbers of participants ranged between 33 and 41.

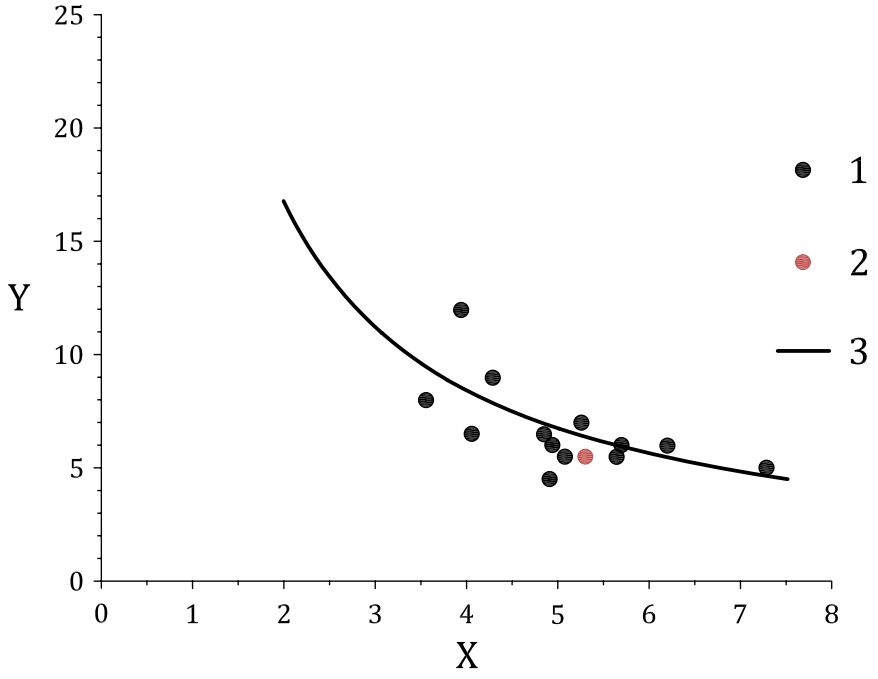
The data processing has been carried out under quality assurance following the requirements of the ISO 5725-2 and covered by accreditation (see Figures H.1 and H.2)



Key

Y	$C_{V,R}$%	1	interlaboratory comparaisons between 2008 and 2014
X	GU per test portion (in log unit)	2	evolution model

Figure H.1 — Changing values of coefficient of variation of reproducibility ($C_{V,R}$%) depending on the number of GU per test portion (log unit) — *Legionella* spp.



Key

Y	$C_{V,R}$%	1	interlaboratory comparaisons between 2008 and 2014
X	GU per test portion (in log unit)	2	test detailed in the example
		3	evolution model

Figure H.2 — Changing values of coefficient of variation of reproducibility ($C_{V,R}$%) depending on the number of GU per test portion (log unit) — *Legionella pneumophila*

Table H.1 — Example of precision observed in an interlaboratory comparison performed in September 2013

	Mean value observed by the laboratories m[a]	**95 % Confidence interval of the mean**	**Repetability[b]**	**Reproducibility[b]**
Quantification of *Legionella pneumophila*	$1{,}98 \times 10^5$	$[1{,}52 \times 10^5 ; 2{,}57 \times 10^5]$	2,3	7,1

[a] In number of GU per 200 ml.

Note that 200 ml correspond to the volume filtered. If 1 L was filtered, the unit would be in UG per litre; the repetability and reproducibility would not be modified.

[b] Expressed as the maximal ratio which is to be expected (at 95 %) between two results from two different laboratories.

If A and B are two results from two different laboratories (by convention $A < B$); a value of 7,1 means that there is 95 % of chance that $\frac{B}{A} \leq 7{,}1$.

The standard deviation of dispersion of the data after log transformation ($s_{\log}$) can be calculated from the value (v), using the following formula: $s_{\log} = \frac{\log(v)}{2{,}8}$; with $v = 7{,}1$; $s_{\text{in log}} = \frac{\log(7{,}1)}{2{,}8} = 0{,}30$.

In Figure H.1, $C_{V,R}$% versus mean value observed by the laboratories is presented graphically (in number of GU per test portion) according to the following calculation: $C_{V,R}\% = \frac{s_{\log}}{m_{\log}} \times 100$.

Then, for the cited example, $m_{\log} = \log(198\,000) = 5{,}297$and $C_{V,R}\% = \frac{0{,}30}{5{,}297} \times 5{,}7\,\%$.

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