
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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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 12869 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Introduction

This Technical Specification specifies a method for the detection and quantification of *Legionella* species (spp.) and *Legionella pneumophila* (*L. pneumophila*) in water using a quantitative polymerase chain reaction (qPCR).

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 Technical Specification 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).

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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)

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

1 Scope

This Technical Specification 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 Technical Specification are given for information only. Any other technical solutions complying with the performance requirements are suitable.

NOTE For performance requirements, see Clause 10.

This Technical Specification is intended to be applied in the bacteriological investigation of all types of water (both hot and cold), 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.

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 Technical Specification is applicable to all types of water. However, some additives, e.g. 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 live or dead cells.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 and definitions

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

3.1

Legionella

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

NOTE rRNA is the abbreviation of ribosomal ribonucleic acid.

3.2

Legionella

<phenotype definition> genus of Gram-negative bacteria normally capable of growth in not less than 2 days on buffered charcoal yeast extract agar containing L-cysteine and iron(III), and forming colonies, often white, purple to blue or lime green in colour

NOTE Some species fluoresce under long-wavelength UV light. The colonies have a ground-glass appearance when viewed with a low power stereomicroscope. With a very few exceptions, growth does not occur in the absence of L-cysteine.

3.3

Legionella pneumophila

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

NOTE 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.

3.4

Legionella pneumophila

<phenotype definition> species belonging to the *Legionella* genus giving a positive reaction in the presence of an anti-*L. pneumophila* serum

NOTE Within *L. pneumophila* at least 15 different groups can be distinguished on the basis of serology. The replication in the environment takes place through intracellular parasitic growth in protozoa.

3.5

deoxyribonucleic acid

DNA

genetic material of living organisms consisting of generic and very specific parts

3.6

polymerase chain reaction

PCR

enzymatic procedure whereby a specific DNA fragment is replicated by a cyclical iterated process of denaturation, annealing of specific primers and DNA synthesis

3.7

amplification

DNA replication

increase of DNA fragments or amplicons as a result of the PCR reaction

3.8

PCR product

PCR amplicon

DNA that is synthesized by the PCR

3.9

quantitative PCR

qPCR

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

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

3.10 **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 The C_t value is the intercept of the line that represents the DNA concentration of a sample with the fluorescent base line.

3.11**reverse primer
forward primer**

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

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

3.12**probe**

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

3.13**Taq DNA polymerase**

polymerase obtained from the bacterium *Thermus aquaticus* that lives in hot springs and geysers

NOTE 1 This thermo-stable polymerase is used for the DNA synthesis in the PCR.

NOTE 2 The use of hot-start polymerase is possible to avoid false-positive results.

3.14***Legionella* spp. genome unit**

GU

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

3.15**detection limit of the qPCR**

LD_{qPCR}

lowest number of genome units that give a positive result in the qPCR with 90 % confidence

3.16**quantification limit of the qPCR**

LQ_{qPCR}

lowest number of genome units that can be quantified with an accuracy less than or equal to $0,15\log_{10}$ unit

3.17**macrophage infectivity potentiator gene**

mip gene

gene present in *Legionella* spp. which is essential for the infection of the host (protozoa) and macrophages (humans)

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

3.18**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 The PCR inhibition control should reveal any inhibitor presence in the sample DNA extract.

NOTE 2 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.

3.19

recovery

efficiency of the DNA extraction

3.20

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

NOTE 2 For the WDCM catalogue, see Reference [3].

3.21

reference material

ready-to-use calibrated DNA solution connected to the *L. pneumophila* DNA primary standard (3.20)

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

3.22

amplification series

set of PCR amplifications run while using the same PCR reagent batches, same materials, and same instruments

3.23

working calibration solutions

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

NOTE The procedure is specified in 7.4.

4 Principle

The detection and quantification of *Legionella* spp. by PCR is 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 with all the necessary precautions. The sampling conditions shall be indicated on the test report if they are known. Perform the sampling in accordance with ISO 19458.

Preferably begin the investigation immediately after the sampling. If samples are delivered to the laboratory within 24 h after sampling, they can be shipped at room temperature. However, if the delay between sampling and arrival to the laboratory is longer than 24 h, the shipment shall be performed at $+5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$.

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

For samples from oxidizing biocide-treated water, the sterile container used for collection shall in addition contain a sufficient quantity of sterile sodium thiosulfate to neutralize the oxidizer (e.g. at a concentration of 20 mg/l).

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 spaces, 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;
- a procedure for eliminating DNA traces shall be implemented in event of accidental contamination of the premises or apparatus;
- regular quality controls checks shall be used to demonstrate the effectiveness of maintenance procedures with the objective of ensuring that there is no contaminating legionella DNA (see 11.4).

6.2 Staff

All personnel who perform aspects of the testing procedures shall be trained to work with PCR and microbiology as appropriate.

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

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

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 amplification.

If automated machines are used, then certain activities can be grouped together in the same area. In all cases, check that there is no contamination (see 11.4).

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 General

Usual laboratory equipment, and in particular the following.

6.4.1.1 PCR hood.

6.4.1.2 Centrifuge.

6.4.1.3 Water bath.

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

6.4.1.5 Consumables. All consumable materials used should be free from DNA or if not then legionella DNA free.

EXAMPLE Filter funnels can be:

- delivered sterile;
- sterilized in an autoclave or oven;
- if made of metal, flamed prior to use.

6.4.2 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 a membrane containing cellulose.

6.4.3 Extraction and PCR (detection and quantification)

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

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

Careful consideration should be given to the apparatus and consumables specified in 6.4.3.2 to 6.4.3.4.

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 Thermoblocks (recommended) to prevent contamination by aerosols.

6.4.3.4 PCR hood, 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 or if not then legionella DNA free.

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

Follow suppliers' recommendations for storage and handling of reagents.

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 within a day of their use.

7 Procedure

7.1 Concentration

Filter as large a volume of the sample as practicable (usually 1 l) to concentrate the bacteria. Record the volume (V) of sample filtered. This is required to calculate the results (see Clause 8). The limit of detection, LD_{meth} (see 10.5) and limit of quantification, LQ_{meth} (see 10.4.4), 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.

7.2.2 Protocols

The DNA is directly extracted on the filter. It is recommended that the whole concentrate be treated.

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

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

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

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

7.2.3 Stability of DNA extracts

DNA extracts shall be stored at $5\text{ °C} \pm 3\text{ °C}$ and analysed within 24 h of preparation. Any longer storage at this temperature requires validation.

DNA extracts can be frozen below -18 °C for several months; these storage conditions shall 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 by two additional primers.

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) will have been defined and optimized. Once these parameters have been established, the performance of the method shall be evaluated (see Clause 10).

The PCR amplification shall include controls described in Clause 11 (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:

- theoretically by homology research using appropriate software in the main databases such as NCBI Genbank (Reference [1]) or EMBL Nucleotide sequence database (Reference [2]);
- 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 10.2. 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 described as a pathogen for humans (described and referenced at the WDCM).

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 10.2).

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).

These preparations are given as examples and shall be validated according to Clause 10.

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

Sequence 5'-CCGATGCCACATCATTAGC-3'

TE buffer (6.5.3.2) diluted 10 times.

LpneuF is prepared 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 Reverse primer *L. pneumophila*: LpneuR, with the following composition.

Sequence 5'-CCAATTGAGCGCCACTCATAG-3'

TE buffer (6.5.3.2) diluted 10 times.

LpneuR is prepared as in a similar fashion to LpneuF (7.3.2.2).

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

Sequence 5'-TGCCCTTTAGCCATTGCTTCCG-3'

Label 5': Fluorophore (carboxyfluorescein, FAM)

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

TE buffer (6.5.3.2)

LpneuP is prepared as follows. Resuspend the freeze-dried probe in the 10-fold diluted TE buffer to a final concentration of 100 µmol/l. Store the stock solution below -18 °C. Dilute the stock solution with TE-buffer (6.5.3.2) diluted 10 times to make a working solution of 10 µmol/l. Store the working solution for up to six months below -18 °C.

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

5'-CCGATGCCACATCATTAGCTTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCCGATTTGGGGA
AGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAAGGCATGCAAGACGCTA
TGAGTGGCGCTCAATTGG-3'

7.3.3 Amplification mix preparation

Ideally, prepare the reaction mixtures immediately before use. If the reaction mix is stored, then its stability requires validation by performing a verification of the linearity of the calibration function after storage (see 10.3). This validation shall meet the criteria defined in 10.3.4.

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.

The composition of a qPCR mix is given in Table 2. This composition is given as an example and shall be validated according to Clause 10.

Mix the components in the proportion indicated in Table 2. Prepare the PCR mix just before use.

7.4 Quantitative detection

7.4.1 General

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

The specificity of the quantitative PCR shall be guaranteed 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 11.2).
- 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 11.6).

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.

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 (10.3). 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 10.3) 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 — Composition of the qPCR mix

Reagents	Volume per sample μl	Final concentration
BSA for PCR applications	5	0,4 μg/μl
Taq polymerase (3.13) ^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
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 10.6.		
^a The volume required depends on the concentration in the stock solutions and can vary with supplier.		

7.4.2 Protocol

7.4.2.1 Introduction

The thermocycler programme 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 programme shall be adapted according to the model and the type of the thermocycler and shall be validated according to the requirements stated in Clause 10.

Table 3 — Temperature and time programme 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

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 used 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 11.2). 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 11.3).

7.4.2.3 Real-time quantification

Aside from the fact that real-time thermocyclers can detect amplification products on site, they are also particularly appropriate for quantitative PCR. The detection systems enable the limits associated with the plateau effect to be circumvented by directly measuring the quantity of amplicon synthesized during the exponential amplification phase. These processes involve extremely sensitive fluorescent emission quantification and detection systems. The principle currently used for calibration is based on quantification of specific amplicons using at least one internal fluorescent labelled probe. Quantification is based on the determination the cycle threshold, C_t , 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 10.3).

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. pneumophila* is expressed as "720 GU/l of *L. pneumophila*".

9 Test report

This test report shall contain at least the following information:

- the test method used, together with a reference to this Technical Specification (ISO/TS 12869:2012);
- all the information required to identify and describe the sample;
- sampling date and conditions;
- the filtered volume of the sample;

- e) the results expressed as described in Clause 8;
- f) any details not included in this Technical Specification that may have an effect on the results.

Table 4 — Expression of results

Number <i>N</i> GU/PCR well	DNA dilution	Reported result GU/l	Comment
$N < 1$	1	$< \frac{LD_{qPCR} F}{V}$	<i>Legionella</i> ^a not detected
	<i>d</i>	$< \frac{LD_{qPCR} dF}{V}$	DNA dilution due to the presence of PCR inhibitors <i>Legionella</i> ^a not detected
$1 < N < LQ_{qPCR}$	1	$< \frac{LQ_{qPCR} F}{V}$	<i>Legionella</i> ^a detected below the limit of quantification
	<i>d</i>	$< \frac{LQ_{qPCR} dF}{V}$	DNA dilution due to the presence of PCR inhibitors <i>Legionella</i> ^a detected below the limit of quantification
$N > LQ_{qPCR}$	1	$\frac{NF}{V}$	<i>Legionella</i> ^a quantitatively detected
	<i>d</i>	$\frac{NdF}{V}$	DNA dilution due to the presence of PCR inhibitors <i>Legionella</i> ^a quantitatively detected
$N > C$	0	$> \frac{CF}{V}$	<i>Legionella</i> ^a detected above the limit of quantification ^b
	<i>d</i>	$> \frac{CdF}{V}$	DNA dilution due to the presence of PCR inhibitors <i>Legionella</i> ^a detected above the limit of quantification ^b
<i>N</i> average number of GU/PCR well LD_{qPCR} limit of detection determined according to 10.5 LQ_{qPCR} limit of quantification determined according to 10.4 <i>C</i> upper value of the calibration range determined according to 10.3 <i>F</i> conversion factor (No. of genome units per well to No. of genome units per litre) <i>d</i> DNA dilution factor <i>V</i> volume, in litres, of sample filtered ^a According to the PCR system, specify <i>Legionella</i> spp. or <i>L. pneumophila</i>. ^b In this case, the quantification can be obtained after DNA dilution.			

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

10.1 General

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 10.

For third party validated commercial methods that fulfil the requirements given in Clause 10, manufacturer's instructions shall be accurately followed.

NOTE For primary validation, all requirements stated in Clause 10 are applicable. For method verification, (secondary validation), simplified requirements can be used for the validation of the correct implementation in the laboratory of any third party fully validated method (see Annex G).

10.2 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 humans.

- Perform inclusivity tests on DNA extracts containing about 100 GU/PCR well.
- Inclusivity list (microorganisms to test for a *Legionella* spp. method): *L. anisa*, *L. birminghamensis*, *L. bozemanii* 1 and 2, *L. cherrii*, *L. cincinnatiensis*, *L. dumoffii*, *L. erythra* 2, *L. feeleei* 1 and 2, *L. gormanii*, *L. hackeliae* 1 and 2, *L. jordanis*, *L. lansingensis*, *L. longbeachae* 1 and 2, *L. maceachernii*, *L. micdadei*, *L. oakridgensis*, *L. parisiensis*, *L. pneumophila* 1 to 15, *L. sainthelensi* 1 and 2, *L. tucsonensis*, *L. wadsworthii*.
- Inclusivity list (microorganisms to test for a *L. pneumophila* method): 15 serogroups from the species.
- Perform exclusivity tests on DNA extracts containing at least 10 000 GU/well.
- Exclusivity list (tested microorganisms recognized as not belonging to *Legionella* genus and/or being phylogenetically close). At least the following list shall be tested: *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Burkholderia cepacia*, *Clostridium* spp., *Enterobacter aerogenes*, *Escherichia coli*, *Flavobacterium* spp., *Klebsiella oxytoca*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia marcescens*, *Stenotrophomonas maltophilia*.
- Exclusivity list (tested microorganisms recognized as not belonging to *L. pneumophila* species): *L. anisa*, *L. bozemanii*, *L. dunmoffii*, *L. gormanii*, *L. jordanis*, *L. longbeachae*, *L. micdadei*, *L. parisiensis*, *L. tucsonensis*.

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

10.3 Verification of the calibration function of the quantitative PCR phase

10.3.1 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.

10.3.2 Calibration curve verification principle

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

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

The parameters of the calibration curve are initially determined by following the evaluation protocol described in 10.3.3. A statistical analysis (see 10.3.4) is then performed in order to:

- determine the equation for the line (see 10.3.4.1);
- verify the linear regression model (see 10.3.4.3).

10.3.3 Calibration curve evaluation protocol

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

Prepare a range of p levels of concentrations of *L. pneumophila* genome units [preferably prepared from the primary standard and, in any case, prepared from *L. pneumophila* (WDCM 00107)], p being at least equal to 4, for example, 25, 250, 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 10.4). 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 5.

Perform the calculations as indicated in the Table 5.

Table 5 — Formatting of results and calculations

Level x_i	$x_1 = \text{LQ}_{\text{PCR}}$	$x_2 = 10\text{LQ}_{\text{PCR}}$	$x_3 = 100\text{LQ}_{\text{PCR}}$	$x_4 = 1\,000\text{LQ}_{\text{PCR}}$	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 number of <i>L. pneumophila</i> genome units per PCR well (the values of x_i levels are given as examples) x'_i logarithm of x_i $y_{i,j}$ Ct value measurement at level i ($i = 1 \dots p$) and row j ($j = 1 \dots k$) k number of repetitions per level i ($k \geq 5$) p number of levels ($p \geq 4$)						

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

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

See Annex C for a full calculation example.

10.3.4 Analysis of the results

10.3.4.1 Estimation of the regression curve

The regression curve is given by Formula (2):

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

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 the following calculations:

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

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

Proceed to the following calculations in order to determine the slope a . The variance of x'_i , $V_{x'_i}$, is given by

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

and the covariance of $x'y$, $\sigma_{x'y}$, by

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

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

$$a = \frac{\sigma_{x'y}}{V_{x'_i}} \quad (7)$$

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

$$\bar{x}' = \frac{\sum x'_i}{N}$$

and on the ordinate

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

Consequently,

$$\bar{y} = a\bar{x}' + b$$

and therefore

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

10.3.4.2 Estimate and verification of the efficiency

Efficiency assesses the yield of the PCR reaction.

Efficiency e is calculated using Formula (8):

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

Efficiency shall have a value between 75 % and 125 %. Consequently, the value of the slope, a , shall be between -4,115 and -2,839.

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

10.3.4.3 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 follows:

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

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

To do this, proceed to the calculations given in Table 6.

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 $0,15 \log_{10} \text{unit}$, the model shall not be validated. In this case, if more than four levels were tested, the data analysis shall be rerun with either the lowest level value (x_1) or the highest level value (x_p) removed, in order to validate a part of the linear range.

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

Table 6 — 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, $\overline{x'_i} - x'_i$	$\overline{x'_1} - x'_1$	$\overline{x'_2} - x'_2$	$\overline{x'_3} - x'_3$	$\overline{x'_4} - x'_4$	$\overline{x'_p} - x'_p$
$s'_i = \sqrt{\frac{\sum_{j=1}^k x'^2_{i,j} - \left[\left(\sum_{j=1}^k x'_{i,j} \right)^2 / k \right]}{k-1}}$	s'_1	s'_2	s'_3	s'_4	s'_p
$E_{lini} = \sqrt{s'^2_i + \left(\overline{x'_i} - x'_i \right)^2}$	E_{lin1}	E_{lin2}	E_{lin3}	E_{lin4}	E_{linp}
$U_{lini} = E_{lini} t_{k-2}$	U_{lin1}	U_{lin2}	U_{lin3}	U_{lin4}	U_{linp}
Theoretical x'_i value calculated using the following equation $x'_i = \log x_i$ $x'_{i,j}$ value calculated using the standard curve from the measurement value $y_{i,j}$ $\overline{x'_i}$ average of $x'_{i,j}$ s'_i standard deviation of values $x'_{i,j}$ with $k - 1$ degrees of freedom E_{lin} accuracy of linearity U_{lin} expanded uncertainty of linearity t_{k-2} value given by the Student table for $k - 2$ degrees of freedom at a risk of 5 % (see Annex D)					

10.3.5 Use of the calibration curve

For each measurement $y = C_t$ of a sample, use the standard curve formula to obtain x' by inverse calibration:

$$x' = \frac{y - b}{a} \quad (10)$$

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 6.

NOTE If the associated standard deviation, s' , is greater than 0,15, the uncertainty of the sample measurement is greater than the uncertainty estimated during the initial method characterization.

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

$$x = 10^{\overline{x'}} \quad (11)$$

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

10.4.1 Principle

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

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

10.4.2 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 11.2). 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.

10.4.3 Analysis of results

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

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

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 equation;

k is the number of measurements.

Calculate the bias using Formula (13):

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

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

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

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

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):

$$U_{LQ} = E_{LQ} t_{\text{tab}} \quad (15)$$

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 7.

Table 7 — 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 7, $E_{LQ} = 0,11$, therefore $E_{LQ} \leq 0,15$, and the limit of quantification at 25 GU is validated.

10.4.4 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):

$$LQ_{\text{meth}} = \frac{LQ_{\text{PCR}}^F}{V} \quad (16)$$

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.

10.5 Verification of the PCR limit of detection (LD_{qPCR})

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

Check that at least 90 % of the results for the targeted LD_{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 11.2).

10.6 Recovery method

10.6.1 Principle

The recovery study shall be carried out on sterile 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 $-0,6\log_{10}\text{unit}$ and $+0,3\log_{10}\text{unit}$.

10.6.2 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 lysates of the mother suspension: simultaneously apply the lysis protocol of the method (lysis solution and physical conditions such as temperature, time, shaking) to three test portions of the mother suspension. The minimum test portion volume is 100 μl introduced into the normal volume of lysis solution (the ratio between the volume of the lysis solution and the test portion volume shall be at least 3). At the end of the lysis, the three unpurified DNA extracts thus obtained shall be, if necessary, diluted so as to remove any lysis reagent-associated PCR inhibition and then, quantified by PCR.

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

Simultaneously create spiked suspensions, from the mother suspension, so as to obtain the targeted concentration levels, i.e. prepare a range of dilutions from the mother suspension (e.g. 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} designated d_1 , d_2 , d_3 , d_4 , and d_5 , respectively). The serial dilutions shall be carried out at 10^{-1} in tryptone salt at 9 ml tryptone salt to 1 ml bacterial suspension. Each tube shall be homogenized by mechanical shaking (approximately 10 s). The dilution levels 10^{-3} (d_3) and 10^{-5} (d_5) correspond, respectively, to approximately 10^6 GU/ml and 10^4 GU/ml. Prepare two spiked samples by inoculating a minimum volume of 100 μ l (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.

10.6.3 Calculations

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

$$\log_{10} \eta_x = B - A + D + \log_{10} \frac{1\,000}{V_{pe}} \quad (21)$$

where

$\log_{10} \eta_x$ is the decimal logarithm of recovery for sample x ;

A is the reference value for the concentration of the mother suspension, expressed as a decimal logarithm of the number of genome units per millilitre;

V_{pe} is the volume of the spiking suspension, in microlitres, μ l;

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.

10.7 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 do this, the recovery for each type of matrix to be tested shall be determined by the laboratory (e.g. cooling tower water, potable water, surface water, waste water) and followed over time (optional control charts). Follow the protocol described in 10.6, 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 10.6.

10.8 Measurement uncertainty of the whole method

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

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

The bias is estimated by the average recovery value for all the matrices (10.6 and 10.7). The precision is estimated through the recovery variance, using all the values obtained during the initial validation of the method (10.6), the robustness study (10.7), and the monitoring of recovery over time (11.4).

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 8 (see example in Annex F).

Table 8 — Calculation of uncertainty from recovery values

Sample No.	Matrix	Level tested	Sample recovery
$x = 1 \dots n$ 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 ($\bar{\eta}_x$)			$\frac{\sum_{x=1}^n \eta_x}{n}$
Variance (s^2)			$s^2 = \frac{\sum_{x=1}^n \eta_x^2 - \left[\left(\sum_{x=1}^n \eta_x \right)^2 / n \right]}{n - 1}$
Overall expanded uncertainty, U_{overall}			$U_{\text{overall}} = 2 \times \sqrt{\bar{\eta}_x^2 + s^2}$

11 Quality controls

11.1 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.

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

11.2.1 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 11.3).

The working calibration solutions (whether or not supplied in a commercial kit) shall be connected to the primary standard at least once a year (see 11.2). 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.

11.2.2 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.

11.2.3 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 0,15, the slopes and therefore the efficiencies are not equivalent. Connection is not possible.

If this value is less than or equal to 0,15, the slopes and therefore the efficiencies are equivalent.

- b) Readjustment of the working calibration solution.

If the slopes are equivalent, calculate the mean of the deviations.

If the absolute value of this mean is greater than 0,15, make another precise calibration solution (zero bias) by dilution from the stock solution.

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

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

An example of connection of the working calibration solution to the primary standard is given in Table 9.

Table 9 — Example of connection of the working calibration solution to the primary standard

Reference range						
Level tested log ₁₀ (GU)	Obtained CTs (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 CTs (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):

$$|\text{calibration error } [\log_{10}(25\ 000) - \log_{10}(25)]| = |0,03 - (-0,01)| = 0,04 \leq 0,15$$

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 $0,15\log_{10}$, no calibration correction is necessary for the calibration solution.

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

11.3 Monitoring of the performances

11.3.1 Calibration performances

The following shall be monitored:

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

The calculated value shall correspond to the reference value $\pm 2 \times 0,15 \log_{10} \text{unit}$.

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

11.3.2 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 $2 \times 0,15$. Laboratories may tighten the limits set by increasing the number of range points.

11.4 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 10.6. 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 (10.6).

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 extractions. The negative control sample shall be the last sample following filtration of all the other samples in the series. The negative control of the method is used to monitor the working environment.

For methods that have been fully validated by a third party, the manufacturer's instructions shall be thoroughly followed for the interpretation of the positive or negative controls and PCR reagent blank (see 11.5).

11.5 PCR reagent blank

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

The negative control (11.4) 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 PCR blank with a C_t value greater than the C_t value of 1 GU shall not be considered as positive.

11.6 Inhibition control

11.6.1 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 11.6.2), or a plasmid or an oligonucleotide (see 11.6.3).

11.6.2 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 CT) are parallel, then perform interpretation according to Table 10.

Table 10 — 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 <i>Legionella</i> spp. or <i>L. pneumophila</i> 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 <i>Legionella</i> spp. or <i>L. pneumophila</i> 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			

11.6.3 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 11 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 11 — Interpretation of the inhibition control when this control is a plasmid or an oligonucleotide

Multiplex amplification		Interpretation
Specific <i>Legionella</i> spp. or <i>L. pneumophila</i> sequence	Inhibition control	
+	Complying	<i>Legionella</i> spp. or <i>L. pneumophila</i> DNA present
+	Not complying ^a	<i>Legionella</i> spp. or <i>L. pneumophila</i> DNA present Partial inhibition or competition, the sample DNA extract shall be diluted until a positive inhibition control is obtained.
–	Complying	No <i>Legionella</i> spp. or <i>L. pneumophila</i> 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.		

Annex A (informative)

Example of protocol for producing a quantitative standard DNA solution

Prepare a culture in a liquid medium (BCYE) at $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ from a colony of *L. pneumophila* (WDCM 00107) isolated on the selective medium.

Measure the optical density at 600 nm. The exponential growth phase is obtained for an optical density value of $0,5 \pm 0,1$.

Extract the DNA according to the laboratory's protocol.

After purification of the DNA and treatment with RNase (to degrade the residual RNA), measure the quantity and quality of the *L. pneumophila* DNA 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 shall 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):

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

where

[DNA] is the concentration of DNA solution (purified) expressed in milligrams per millilitre;
 A(260) is the optical density measured at 260 nm.

Dilute the DNA extract 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).

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

Annex B (informative)

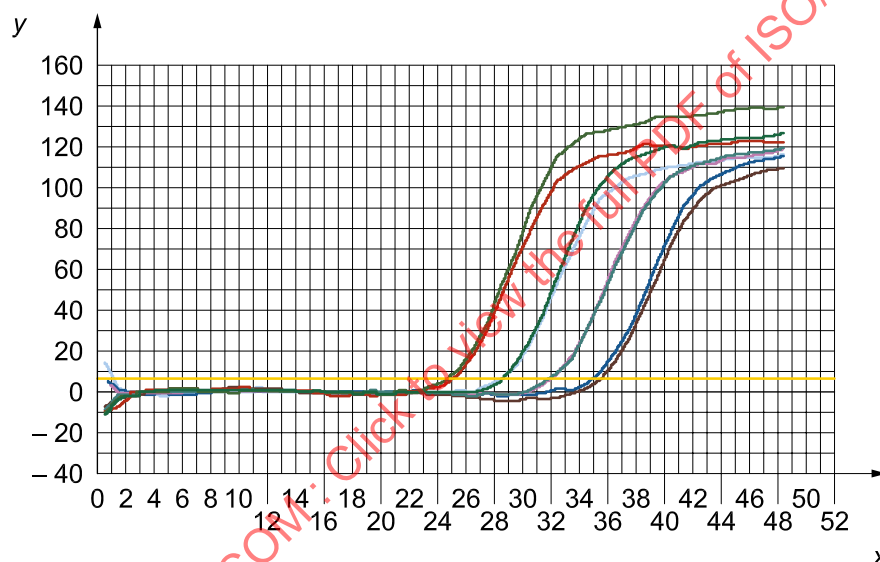
Example of method for determining the cycle threshold

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.

At the end of PCR, the profiles below are obtained (see Figure B.1).



Key

y PCR baseline subtracted RFI
 x cycle

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 n_{iGU} is the initial number of genome units, can be determined from the C_t of the range points.