Technical Specification

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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) —

Part 2: On-site methods

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) —

Partie 2: Méthodes sur site

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Contents

Page

Foreword

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

A list of all parts in the ISO 12869 series can be found on the ISO website.

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

ISO/TS 12869 provides the guidelines, minimum requirements and performance characteristics intended to guarantee that the quantification of *L. pneumophila* or *Legionella* spp. by amplification of specific DNA sequences (PCR) and real time detection of specific DNA sequences (PCR) and real-time detection of specific fluorophores is reproducible between methodologies completed by different laboratories.

Similar to ISO/TS 12869, this document specifies a method to determine recovery of the bacteria and subsequent DNA amplification (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) —

Part 2: On-site methods

1 Scope

This document provides the guidelines, minimum requirements and performance characteristics intended to guarantee that manufactured systems intended for on-site/field use (i.e. outside the laboratory) provide reliable and reproducible results.

This document specifies the requirements for technologies that enable on-site detection and quantification of *Legionella* spp. and *L. pneumophila* using a quantitative polymerase chain reaction assay (qPCR). It specifies general methodological requirements, performance evaluation requirements and quality control requirements. This document is intended to be used by manufacturers of these technologies so that they produce detection systems that end users can operate safely and effectively. End users will be guided by this document to adhere to manufacturer's instructions, to ensure user competency and to perform the necessary controls.

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

NOTE For validation and 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 background microorganisms 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 millilitre (or litre) of sample.

Although the method described in this document is applicable to all types of water, 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.* However, there are on-site qPCR methodologies which are able to distinguish intact bacteria from free DNA.

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 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

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

ISO/TS 12869:2019, *Water quality — Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)*

ISO 11731, *Water quality — Enumeration of Legionella*

3 Terms, definitions, symbols and abbreviated terms

3.1 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 12869 and the following apply.

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

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

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

3.1.1
***Legionella* spp.**
several species of *Legionella*, including *L. pneumophila*

3.1.2
polymerase chain reaction inhibition control
PCR inhibition control
materials and processes used to assess if the sample DNA extract contains (a) PCR inhibitor(s)

Note 1 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.

3.1.3
bacterial recovery
evaluation of the reported quantity of bacteria by the *on-site qPCR* (3.1.7) system when a known quantity of reference material is tested

3.1.4
working calibration solution
L. pneumophila DNA calibrated solutions, derived from a standard solution, for which accuracy is determined by an independent method (e.g. digital droplet PCR) used to establish the calibration curve

3.1.5
negative control of the method
control for monitoring the whole process in this method (from filtration to extraction to qPCR)

3.1.6
no template control
NTC
control for monitoring qPCR reagent amplification

3.1.7
on-site qPCR
qPCR testing that can occur immediately after sample collection, such that sample preservation is not required (e.g. sodium thiosulfate)

Note 1 to entry: On-site qPCR is validated for use by non-laboratory personnel that have been trained in the procedure.

3.1.8
concentration device
device that prepares a water sample for qPCR amplification

Note 1 to entry: This kind of device is designed such that it can be used safely and effectively by non-laboratory trained personnel.

3.1.9
threshold cycle
C_t
Note 1 to entry: 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 2 to entry: The C_t value is the intercept of the line that represents the DNA concentration of a sample with fluorescent base line.

3.1.10
genome unit
GU
Note 1 to entry: unit representing a single copy of bacterial genomic DNA

3.1.11
graphical user interface
GUI
on-screen controls of the testing equipment which can describe sample concentration and analysis procedure

Note 1 to entry: The interface is designed such that it can be used and understood by non-laboratory personnel that have been trained in the procedure.

3.1.12
end user
operator
individual who performs the assay on the test system

3.1.13
critical task
step in the on-site test workflow that can lead to a hazardous situation, such as an incorrect test result and/or injury to the test *operator* (3.1.12), if performed incorrectly

3.1.14
batch
manufacturing production run used to generate one or more lots of finished goods

3.2 Symbols and abbreviated terms

C_t	threshold cycle
D_{opt600}	optical density at 600 nm
L_{DqPCR}	(detection limit of the qPCR) lowest number of genome units that give a positive result in the qPCR with 90 % confidence
L_{QqPCR}	(quantification limit of the qPCR) lowest number of genome units that can be quantified with an accuracy less than or equal to 0,20 $\log_{10}$ unit
V_b	volume of the bacterial sample in the reaction
V_f	final volume of the reaction
V_w	volume of water in the reaction
BCYE	buffered charcoal yeast extract
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
GMP	good manufacturing practice

GU	genome unit
GUI	graphical user interface
HFT	human factors testing
NTC	no template control
OD	optical density
PPE	personal protective equipment
QC	quality control
UNG	uracil-DNA N-glycosylase

4 Principle

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

— concentration of bacteria from water samples by the concentration device;

— recovery of the bacteria from the concentration device and transfer of the bacteria to a vessel or apparatus in which bacterial lysis and DNA extraction occur; PCR can then proceed in the same apparatus or an additional step can be required to transfer the extracted DNA to a PCR reaction;

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

5 Sampling

Sampling shall be in accordance with ISO 19458, however one of the main advantages of on-site testing is the ability to test samples immediately after collection, thus mitigating the known effects of time on sample quality (see Reference [1]). The manufacturer shall indicate to the end user the acceptable holding times between sample collection and analysis. These time intervals can vary between water sources (e.g. potable water without biocides present versus cooling tower samples that contain biocides). The manufacturer shall validate these holding times, which will be provided in the instructions (see 6.2.2).

Sampling conditions (e.g. water treatment, temperature, turbidity, time that water was run prior to sampling) shall be indicated on the test report if they are known. Manufacturers will validate conditions, including temperature and commonly used chemicals (e.g. biocides, neutralizing agents, anti-corrosives) in intended sample types, that are compatible with the testing system. Manufacturers will indicate the compatible sample conditions to end users. Samples shall not be exposed to conditions that the manufacturer has not validated.

Biocides (bactericides or bacteriostatics) are sometimes used, in particular in cooling tower circuits. The presence of biocides, however, can lead to under quantification of the analyte, therefore the presence of biocides shall thus be declared and indicated on the test report if it is known. When inhibition of PCR sufficient to result in under quantification is detected, the test result shall be suppressed and a warning message specifying that interference was encountered shall be provided to the end user. Where appropriate, sample containers shall contain a suitable neutralizing agent (refer to ISO 19458). As it is not always possible to neutralize these products, minimizing the elapsed time between sample collection and analysis is recommended.

Manufacturers shall indicate to end users the need for a sampling plan and refer users to ISO 5667-1 for guidance.

6 General testing conditions

6.1 General

PCR is a sensitive detection method, the results of which can be affected by aerosols, dust and other particles which can contain contaminating DNA. It is therefore essential to physically separate the different stages of the analysis. The on-site qPCR concentration device shall be designed in such a way to prevent this type of contamination.

The principles to be applied are as follows:

— single use concentration device and qPCR reagent;

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

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

The manufacturer and the end users shall fulfil the responsibilities listed in Annex A.

6.2 End users

6.2.1 General

All personnel who perform on-site qPCR shall be provided with instructions to operate the system, as well as training materials as needed. Instructions shall be provided as a physical copy of the instructions, a training video or interactive instructions provided by a graphical user interface (GUI).

The test operators shall wear personal protective equipment required for sample collection as per jurisdictional guidelines. Gloves are required. They shall be disposable and talc-free.

As the qPCR results shall be analysed and interpreted by software and expressed to the user via the GUI in appropriate units (e.g. GU/ml or GU/l), the operator shall not require additional advanced training or experience in PCR data analysis. Likewise, the presence of inhibition shall be determined, via PCR inhibition control, by automated analysis of the data by the software.

6.2.2 Manufacturer's instructions

Instructions shall be provided to end users by manufacturers. The instructions shall include clear and specific information necessary to safely and effectively perform tests on-site. The following topics shall be included in the manufacturer's instructions to end users.

— Intended use: Statement of the test system input material(s) and result output(s) and how the results may be used. The latter shall include a statement notifying the end user to be aware of jurisdictional requirements and how they can affect how the results are used. The intended end user shall also be provided.

— Warnings and precautions: Description of safety measures required to avoid any risk of harm to the operator or other individuals when the test is performed. Risks considered shall include the risk of exposure to aerosolized *Legionella* as well as any critical concerns that can lead to incorrect results or expose the operator to risk (such as do not use if damaged, store consumables as indicated, follow critical steps in the instructions/workflow, etc.). Detailed handling and waste disposal instructions shall also be provided.

— Personal protective equipment (PPE): Description of the appropriate PPE required by users to handle and use the system safely, including protection from the risk of exposure to aerosolized *Legionella*.

- Storage/operating conditions: Temperature, humidity and any other environmental requirements for storage and safe and effective use of the consumables and equipment.
- Training requirements: Clear description of the program that test operators have to complete prior to being considered proficient in the test system. This can range from use of a quick reference guide to in-person training by a manufacturer-trained trainer. All training programs shall be validated by the manufacturer (refer to 6.2.4).
- Workflow instructions for samples and any control tests including:
  - step-by-step workflow instructions,
  - any pertinent warnings around steps critical to test success/accuracy,
  - sample holding times and
  - any other time constraints
  - warnings to users not to open the amplified DNA container.
- Controls: Description of internal and external controls and suggested frequency to perform external controls. The instructions shall also indicate:
  - how to proceed when control tests fail, which may entail calibration or maintenance;
  - that if the control procedures result in a test system being out of control, the results of any tests performed prior to control failure are invalidated; and
  - external control tests may be performed at a frequency based on test frequency (e.g. if several tests are performed per week, weekly control tests can be adequate, while weekly control tests will be unnecessary if testing is performed semi-monthly).
- Troubleshooting: Any troubleshooting procedures including how to perform a test if the sample is inhibitory. This may be the dilution of the sample and/or a decreased sample input into the test system. All troubleshooting procedures, including their usability, shall be validated by the manufacturer.
- Test results: Example test result and interpretation instructions.
- Maintenance and calibration: Any maintenance and calibration procedures required (see 6.7).
- Decontamination: Detailed and validated decontamination procedures that are compatible with the testing equipment, as well as indications of when to perform the decontamination procedures.
- Performance characteristics: Summary of the results of the manufacturer's accuracy and precision validation studies. Any information about substances or circumstances that may interfere with testing, including matrix and sample storage conditions, shall also be included in this section.

6.2.3 Proficiency

Analysis of samples shall only be conducted by personnel having documented proficiency. Proficiency shall be verified through the use of external controls and user proficiency guidelines shall be provided by the manufacturer.

Representative, intended end users (defined by the manufacturer) shall complete the manufacturer's training program (which can involve training by a designated trainer, self-training and/or reading guides or instructions) and then perform tests using the manufactured system with representative test inputs and/or controls. Test results shall be within the accuracy requirements specified in ISO/TS 12869:2019, Clause 9.

6.2.4 Usability validation (human factors testing [HFT])

The manufacturer shall validate the usability of the system by the intended end user. Validation of the usability shall demonstrate that intended users, upon completion of any required training, can perform the

test safely and effectively, by adhering to the manufacturer's instructions. The manufacturer shall perform risk analysis to identify critical steps in the workflow that affect test safety and accuracy. Any critical steps in the test shall be specifically assessed during usability validation. The pass criteria for usability validation shall include successful completion of all critical steps by intended users and adherence to all safety requirements. An example usability validation protocol is provided in Annex B.

6.3 Premises

6.3.1 Manufacturer premises

The following operations shall be physically separated by design:

a) preparation of PCR reagents (reaction mixtures);

b) concentration of samples and/or DNA extraction;

c) PCR amplification.

The preparation of PCR reagents shall be performed following good manufacturing practices (GMPs).

The operating and storage conditions compatible with the test system (equipment and consumables) shall be validated by the manufacturer, who will inform the end user of these requirements.

6.3.2 End user premises

The manufacturer shall validate the operating conditions (e.g. temperature, humidity, illumination) of the test system. The validated operating environment shall represent the expected operating conditions of the intended end user(s) and will be provided to the end user by the manufacturer.

The end user shall perform the assay in an area that conforms to the operating conditions specified by the manufacturer. The end user shall store the equipment and consumables in accordance with the manufacturer's instructions. The records of operating and storage conditions shall be maintained by the end user.

Regardless of the amplicon detection and amplification system used, no sample container (e.g. PCR tube) shall be opened after amplification.

6.4 Apparatus and consumables (excluding reagents)

6.4.1 General

All used consumables shall be free of *Legionella* DNA.

6.4.2 Safety

When working with *Legionella*, manufacturers shall adhere to the following safety warning provided in ISO/TS 12869:

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.

The manufacturer of any device or system used to process *Legionella* bacteria shall provide safety warnings and precautions for the end user regarding the handling of samples potentially containing *Legionella pneumophila*, a pathogen ordinarily requiring level 2 containment. All devices used to process the samples shall be designed to minimize the generation of aerosols and shall inactivate the bacteria once present in a

concentrated form. The following techniques are known to generate aerosols and shall not be included in procedures in which living *Legionella* samples are used:

— pouring or other transfer techniques that produce splashing;

— flipping open caps of containers of *Legionella* samples;

— flicking or shaking containers of *Legionella* samples;

— vortexing containers of *Legionella* samples;

— pipette mixing *Legionella* samples.

6.4.3 Concentration

A concentration device suitable for on-site testing may contain a membrane filter. Such a filter 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. Membrane filters containing cellulose or glass fibre are not suitable and shall not be used. The concentration device shall be designed to be used by non-laboratory trained personnel and validated instructions for use shall be provided.

6.4.4 PCR (detection and quantification)

6.4.4.1 Real-time thermocycler

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

6.4.4.2 Consumables

The consumables shall be free from nucleases, PCR inhibitors and *Legionella* DNA. The design of consumables shall prevent contamination of the testing equipment with the water sample. Regular quality control tests (e.g. external negative control in the form of a no template control [NTC]) performed by the end user (refer to 10.4.2) will verify the absence of contamination. Any decontamination procedures of reusable consumables shall be validated by the manufacturer to have expunged any *Legionella* or *Legionella* DNA to below the detection limits of the testing system.

6.4.4.3 Graphical user interface

The PCR thermocycler, or a separate device, will contain software that will interpret the fluorescent signal from the thermocycler and translate it to the amount of DNA product present in the water source in appropriate units (e.g. GU/ml or GU/l). The GUI will display the result in a format that is clear and unambiguous to the end user and shall alert the user in the event of an unsuccessful test. The GUI will also guide the user through any necessary steps required to execute a successful test.

6.5 Reagents

All reagents used shall be free from nucleases, PCR inhibitors and *Legionella* DNA. PCR grade water shall be used in the manufacture of all solutions.

For on-site testing, reagents shall be dispensed in single use aliquots. Manufacturers of on-site testing reagents shall follow GMPs in order to ensure traceability and viability, including the absence of any contamination, of all reagents.

PCR reaction mixes shall be produced by the manufacturer (see Table 1 for an example of PCR reaction mix components) and shall go through all necessary quality controls before being provided to the user (see 10.4 and 10.5).

6.6 Decontamination of equipment and premises

Manufacturers of on-site testing devices shall minimize the potential for contamination of end user equipment and premises by the design of the system. Any decontamination procedure and the frequency of such a procedure shall be provided in the manufacturer's instructions (see 6.2.2).

In the event of accidental or non-accidental contamination at the manufacturing facility, equipment and premises, refer to ISO/TS 12869:2019, 6.6.

6.7 Maintenance and calibration

Manufacturers shall design testing equipment such that it does not require maintenance and/or calibration; regular external testing and internal quality control systems can ensure that equipment is functioning properly. If equipment requires maintenance and/or calibration, it may be returned to the manufacturer or the procedures may be performed by the end user. Any maintenance and calibration procedures, including the ability of the intended end users to perform said procedures, shall be validated by the manufacturer.

6.8 Treatment and elimination of waste

For the treatment and elimination of waste, refer to ISO/TS 12869:2019, 6.7.

7 Procedure

7.1 Concentration

The system shall concentrate by filtration the minimum volume of sample required to obtain the desired levels of sensitivity in terms of limits of detection, L_{DqPCR}, (see 9.6) and of quantification, L_{QqPCR} (see 9.4). The determination of volume required will be determined by the manufacturer and may depend on the required sensitivity. This volume is directly related to the efficiency of concentration and recovery. The volume, *V*, of the sample filtered shall be used by the GUI to automatically calculate the results.

The volume of sample to be filtered shall be indicated to the user via instructions for use and/or the GUI and/or additional usage guides. The volume tested shall be recorded on the final report when the system used to process samples accepts a range of volumes.

7.2 Bacterial concentration and bacterial recovery

7.2.1 General

Bacterial concentration (see 6.4.2) and bacterial recovery can involve collecting intact bacteria on the surface of a suitable filter while eliminating other components, such as PCR inhibitors, as efficiently as possible. Wash steps will be incorporated to eliminate inhibitors. These wash steps can also remove free DNA that can still be present on the filter surface. In such cases, the validation of the method shall include satisfying this performance requirement. The concentrated bacteria will then be eluted from the filter into a suitable reagent for lysis and genetic amplification by qPCR. All workflow steps shall be clearly described for all analysts, including non-laboratory trained personnel and shall not require access to laboratory equipment or require laboratory expertise.

7.2.2 Protocols

Any necessary steps to prepare the bacteria shall be clearly outlined and designed to be followed by non-laboratory trained personnel. A concentration system can be used to prepare the sample. In this case, the bacterial concentrate can be directly eluted into the subsequent reaction vessel or vessels. Any concentrated bacteria shall be inactivated or handled automatically by the device such that exposure of the operator to aerosolized bacteria is prevented.

7.2.3 Stability of bacterial eluates and DNA

To elute the bacteria, suitable methodologies that maintain the integrity of the bacteria may be used. These procedures shall prevent excessive damage or release of genomic DNA which can result in a loss of quantifiable material. Once eluted, the bacteria can then be transferred to the appropriate assay reagents.

After bacteria elution, the sample can be immediately and directly used for DNA extraction and PCR in a single reaction, which may or may not include DNA isolation. Any DNA isolation and subsequent addition of the isolated material to the PCR reaction shall not require laboratory training by the end user. The time between bacterial elution and qPCR shall be validated by the manufacturer, who shall provide all time and storage constraints, as they pertain to intermediate stability during the workflow, to the end user. Concentrated, active bacteria shall not be stored by the end user.

7.3 DNA amplification by PCR

7.3.1 General

Refer to ISO/TS 12869:2019, 7.3.1.

The PCR amplification shall include PCR inhibition controls described in Clause 10. The design of all other controls (negative, positive and reference material) will be validated by the manufacturer and subjected to QC testing prior to being provided to the end user.

7.3.2 Target sequences, primers and probes

7.3.2.1 General

Refer to ISO/TS 12869:2019, 7.3.2.1.

7.3.2.2 Example primers and probes

For information on example primers and probes, refer to ISO/TS 12869:2019, 7.3.2.2 to 7.3.2.9.

7.3.3 Amplification mix preparation

Prepare the PCR master mix in accordance with GMPs and/or in conformance to jurisdictional manufacturing requirements. An example of a typical PCR reaction mix is provided in Table 1. Master mix shall be stored in defined optimal conditions to maximize stability. Stability requires validation by performing a verification of the linearity of the calibration function after storage (see 9.3) to determine the life of the product. This validation shall meet the criteria defined in ISO/TS 12869:2019, 9.4.3. Third-party master mixes can be commercially available. Any commercially available master mix used in the testing system shall be validated by the manufacturer and shall not require the end user to have laboratory training for its use.

NOTE There are also commercially available PCR master mixes available which can be used for on-site methods.

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.
$MgCl_2$	The final concentration of $MgCl_2$ depends on the dNTP, primer, 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	Refer to ISO/TS 12869:2019, 7.3.2.2, 7.3.2.3, 7.3.2.5 and 7.3.2.6.
Thermostable DNA polymerase	Hot-start Taq DNA polymerase can be used to avoid false-positive results.
Probes	Refer to ISO/TS 12869:2019, 7.3.2.4 and 7.3.2.7.
[a] Depending on their source, some of these components can previously be mixed in the PCR buffer solution.	

An example of a typical qPCR reaction mix is provided in Table 2.

Table 2 — Example of composition of the qPCR mix

Reagents	Volume per batch ml	Final concentration
BSA for PCR applications	5	0,4 µg/µl
Taq polymerase[a]	1	0,1 U/µl
PCR buffer (see Table 1)	5	1×
dNTPs (see Table 1)	1	400 nmol/l
$MgCl_2$[a] (see Table 1)	3	According to the Taq DNA polymerase requirements
Primer LpneuF (refer to ISO/TS 12869:2019, 7.3.2.2)	1	200 nmol/l
Primer LpneuR (refer to ISO/TS 12869:2019, 7.3.2.3)	1	200 nmol/l
Probe LpneuP (refer to ISO/TS 12869:2019, 7.3.2.4)	1	200 nmol/l
Water for PCR applications	make up to 40	
PCR inhibition control shall be added in accordance with 9.6. [a] The volume required depends on the concentration in the stock solutions and can vary with supplier.		

7.4 Quantitative detection

7.4.1 General

This real-time PCR based method shall enable the 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. However, if a multiplex reaction is developed, it shall be validated by the manufacturer to estimate the performance characteristics.

The specificity of the quantitative PCR shall be ensured by using specific hybridization probe(s). To ensure the quality of the quantitative detection, use:

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

b) a PCR inhibition control to reveal the presence of an inhibitor in the sample (see 10.6).

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. When the result is above the L_{QqPCR} of the whole method, the result will be a number of genome units per unit volume (e.g. GU/ml or GU/l). The C_t value is estimated [see b)] during the characterization and the validation of the method (refer to ISO/TS 12869:2019, 9.3.2).

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 then the verification of the method (see 9.3). The sample can, if necessary, be diluted to obtain a concentration situated within this linear response area, as described in 9.3.

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.

7.4.2 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).

Table 3 — Example of a temperature and time program for PCR

Denaturing of DNA and activation of hot-start Taq polymerase	DNA replication	Number of cycles
3 min at 95 °C	20 s at 95 °C 60 s at 60 °C	43[a]
[a] This number of cycles is determined as the intercept of the calibration function +5 in order to be able to observe the amplification profile obtained for 1 GU.		

This program shall be adapted in accordance with the model and the type of the thermocycler and shall be validated in accordance with the requirements of Clause 9.

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.

7.4.2.2 General

Refer to ISO/TS 12869:2019, 7.4.2.2 for the general approach to detect or quantify amplicons.

For on-site qPCR systems, the working calibration range shall be analysed for every batch by the manufacturer. The calibration series shall be verified to be stable by the manufacturer under validated storage conditions.

7.4.2.3 Real-time quantification

For information on real-time quantification, refer to ISO/TS 12869:2019, 7.4.2.3.

7.5 Qualitative detection

For information on qualitative detection, refer to ISO/TS 12869:2019, 7.5.

8 Expression of the results

All calculations to quantify the number of genome units present in a PCR reaction will be performed by the software and will not require interpretation by the user. This information will be further converted to express the concentration of *Legionella* spp. or *L. pneumophila* in the water sample in appropriate units. The testing system shall include internal controls that suppress results when interpretation by the system's result-determining algorithm is unable to provide a result within the validated accuracy.

Express the results in accordance with ISO/TS 12869:2019, Table 3, in number of genome units (GU) of *Legionella* spp. and/or *L. pneumophila* per litre or per millilitre 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 72 GU/ml of *L. pneumophil*a is expressed as "72 GU/ml of *L. pneumophil*a".

For the expression of the results for qualitative detection, refer to ISO/TS 12869:2019, Table 5.

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

9.1 General

The technical criteria and requirements described in Clause 9 shall be used for the characterization and for the validation of on-site qPCR methods.

Any protocol for routine application shall be validated in accordance with the requirements of Clause 9.

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

The performance characteristics of the testing system, minimally including the L_{DqPCR} (see 9.4), L_{QqPCR} of the whole method (see 9.5.4), reproducibility (see 9.7.1) and uncertainty of the whole method (see 9.9) shall be provided by the manufacturer to the end user.

9.2 Inclusivity and exclusivity of probes and primers

For information on the inclusivity and exclusivity of probes and primers, refer to ISO/TS 12869:2019, 9.2.

9.3 Verification of the calibration function of the quantitative PCR phase

For information on the verification of the calibration function of the quantitative PCR phase, refer to ISO/TS 12869:2019, 9.3.

9.4 Verification of the PCR limit of detection, L_{DqPCR}

For information on the verification of the PCR limit of detection, refer to ISO/TS 12869:2019, 9.5.

9.5 Verification of the PCR limit of quantification, L_{QqPCR}

9.5.1 Principle

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

The quantification limit is verified if the accuracy at the quantification limit, E_{LOQ}, is less than or equal to the critical value of 0,20 $\log_{10}$ unit.

NOTE The 0,20 $\log_{10}$ unit value comes from experimental data.

9.5.2 Experimental design

Prepare at least 10 separate dilutions at the targeted L_{QqPCR} value from a DNA solution of *L. pneumophila* derived from the primary standard (refer to ISO/TS 12869:2019, 10.2). Quantify each dilution in accordance with the test system's normal protocol under these intermediate precision conditions (at least on different days and/or by different operators).

9.5.3 Analysis of results

For information on the analysis of results, refer to ISO/TS 12869:2019, 9.3.

9.5.4 Theoretical limit of quantification of the whole method

For information on the theoretical limit of quantification of the whole method, refer to ISO/TS 12869:2019, 9.4.4.

9.6 Recovery/accuracy

9.6.1 Principle

Recovery, a measure of accuracy, is an evaluation of the reported quantity of *L. pneumophila* by the on-site qPCR system when a known quantity of *L. pneumophila* reference material is tested.

This study will be carried out on water samples that are known to be free from *Legionella* DNA. These water samples will be artificially contaminated with dilutions of a stock suspension of *L. pneumophila* (WDCM 00107 or equivalent).

Recovery is assessed using a minimum of nine determinations over a minimum of three concentration levels that cover the range of the calibration curve. Typical concentration levels correspond to the lower, middle and upper ranges of the limit of quantification.

Calculate the recovery by logarithm difference. Recovery shall have a value between $-0{,}3\log_{10}$unit and $+0{,}3\log_{10}$unit.

9.6.2 Protocol for preparation of bacteria

To perform the recovery method, aseptically grow colonies of *L. pneumophila* (WDCM 00107 or equivalent) on an appropriate solid medium (e.g. buffered charcoal yeast extract [BCYE] agar). Aseptically transfer *L. pneumophila* colonies (e.g. five colonies) that are younger than 72 h old into a tube containing 2 ml liquid growth medium (e.g. BCYE).

Grow the stock of *Legionella* bacteria at 37 °C in aerated media (e.g. shaker flask) to an approximate optical density (OD) of 0,5 at 600 nm, which approximately corresponds to a concentration of *Legionella* spp. of 10^9 GU/ml. Based on the optical density at 600 nm of the liquid bacterial stock suspension, prepare a 10^8 GU/ml dilution mother suspension in a diluent suitable for living *Legionella* in accordance with ISO 11731, based on Formulae (1) and (2):

$$V_w = V_f - \frac{10^8 \times V_f}{\frac{D_{opt\,600}}{0{,}5} \times 10^9} \quad (1)$$

where

D_{opt600} is the optical density at 600 nm;

V_w is the volume of water in the reaction, in µl;

V_f is the final volume of the reaction, in µl.

$$V_b = \frac{10^8 \times V_f}{\frac{D_{opt600}}{0,5} \times 10^9} \quad (2)$$

where

V_b is the volume of the bacterial sample in the reaction, in µl;

V_f is the final volume of the reaction, in µl.

Dilute the 10^8 GU/ml dilution mother suspension to achieve the desired target concentrations to evaluate recovery for direct PCR analysis. Serial dilutions shall be carried out in sterile water. Each tube shall be thoroughly mixed prior to preparing the subsequent dilution.

Measure the concentration of genome units in the stock suspension by direct PCR in a minimum of three reactions.

Another method to make suspensions of *L. pneumophila* for recovery experiments is to use a commercially available reference material with a certified amount (expressed in GU) of *L. pneumophila.*

9.7 Precision

9.7.1 General

Precision is a validation of the reproducibility of the results using the on-site qPCR system.

9.7.2 Reproducibility

This study will be carried out on water samples that are known to be free from *Legionella* DNA. The water samples will be artificially contaminated with dilutions of a stock suspension of *L. pneumophila* (WDCM 00107 or equivalent). Preparations of *L. pneumophila* can be prepared according to 9.6.2.

Reproducibility is assessed using a minimum of 10 determinations at the upper and lower limits of quantification concentrations of the system.

Reproducibility results for each concentration shall satisfy the recovery acceptance criteria in ISO/TS 12869:2019, 9.6.1.

9.7.3 Intermediate precision

Intermediate precision evaluates the performance of the on-site qPCR system when variables, such as different operators or different testing times/days, are introduced.

This study will be carried out on water samples that are known to be free from *Legionella* DNA. The water samples will be artificially contaminated with dilutions of a stock suspension of *L. pneumophila* (WDCM 00107 or equivalent). Preparations of *L. pneumophila* can be prepared according to 9.6.2.

Intermediate precision is assessed using a minimum of three determinations over a minimum of two concentrations within the range of the calibration curve for each variable introduced. For example, three operators will test three determinations of all concentrations over 3 d.

The results for all intermediate precision conditions will be pooled by variable for evaluation. All intermediate precision conditions evaluated shall have results that satisfy the recovery acceptance criteria of ISO/TS 12869:2019, 9.6.1.

9.8 Robustness

In this instance, robustness is determined through the characterization of the system's performance when testing representative water samples. Recovery shall not be affected by the type of matrix (e.g. cooling

tower water) to be analysed for bacterial contamination. The effects of matrix storage conditions, including time, humidity and temperature, shall be characterized as part of robustness determination. The effects of matrix composition and sample storage conditions on test robustness shall be included in the performance characteristics provided to the end user by the manufacturer (see 6.2.2).

To perform this, follow the protocol described in 9.6.2, by replacing the appropriate diluent with the matrix to be assessed (using the appropriate volume determined for the on-site qPCR system). The acceptable limits shall be in accordance with the recovery requirements of ISO/TS 12869:2019, 9.6.1.

9.9 Measurement uncertainty of the whole method

Manufacturers shall determine the uncertainty of measurement of the whole method in accordance with ISO/TS 12869:2019, 9.8.

9.10 On-site verification by end user

While manufacturers shall validate system performance, end users shall perform on-site verification to verify that the testing system yields accurate and reproducible results in the end user's environment. The manufacturer shall provide guidelines regarding how to perform the verification, which can utilize external controls. The manufacturer shall also provide the end user with the pass/fail criteria for the verification testing. On-site verification shall minimally include accuracy and reproducibility tests. An example on-site verification protocol is provided in Annex C.

10 Quality control

10.1 General

Quality controls ensure trueness and precision of measurements carried out by the device. Manufacturers shall validate the accuracy and precision of their testing systems, while end users shall verify not only system performance but also user competency using external controls either available commercially or provided by the manufacturer.

Internal controls shall be designed and validated such that if a user fails to correctly perform a critical task in the procedure, the test system will suppress the test result. For example, if a test operator does not perform a critical task that dispenses the concentrated sample into a test reaction, a "low volume" signal triggers suppression of the test result. Internal controls may be multiplexed with *Legionella* detection, but it has to be validated by the manufacturer that the internal control does not interfere with quantification within the quantifiable range of the test system.

Minimum frequencies of controls required when routinely using devices shall be established by the manufacturer. The accumulation of results can allow these frequencies to be modified. The manufacturer shall provide end users with recommended frequencies of external control tests.

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 two levels of standards:

a) a primary standard;

b) working calibration solutions, for which the accuracy will be determined by an independent method (e.g. digital droplet PCR).

Accuracy shall be validated using an independent method (e.g. digital droplet). Moreover, accuracy shall be validated by the manufacturer of the device in the case of any changes to the calibration solutions.

10.2.2 Protocol

To prepare the working calibration solutions, the primary standard is diluted and tested to ensure that the dilutions perform within the previously validated specifications (e.g. C_t). Additionally, accuracy shall be confirmed, by the manufacturer, against an independent method (e.g. digital droplet PCR).

10.2.3 Data analysis

For information on data analysis, refer to ISO/TS 12869:2019, 10.2.3.

10.3 Monitoring performance

Manufacturers of on-site devices shall establish QC acceptance criteria to assess each batch.

Working calibration solutions shall be assessed with each batch as described in ISO/TS 12869:2019, 10.2.

10.4 Positive and negative controls of the method

10.4.1 Positive and negative controls performed by the manufacturer

Manufacturers of on-site testing devices shall, as a positive control, verify the recovery of the system initially as well as re-verify every time there is a change in any part of the system that can affect performance as determined through risk analysis.

Manufacturers of on-site testing devices shall, as a negative control, verify NTC result rate (PCR negatives of DNA-free sterile water) of the system initially as well as re-verify every time there is a change in any part of the system that can affect performance as determined through risk analysis.

10.4.2 Positive and negative controls performed by the end user

10.4.2.1 General

A validated, suggested quality control program shall be provided to the end user by the manufacturer. End users shall periodically perform negative controls (e.g. NTC) and positive controls. The frequency of external controls shall be suggested by the manufacturer and will depend on several factors, including test frequency, personnel changes, suspected issues, changes to the testing premises.

10.4.2.2 External negative controls performed by the end user

The manufacturer shall provide the end user with a procedure to perform a test that does not result in a positive result. This may be performed using samples provided by the manufacturer or commercially available materials, such as distilled water, specified by the manufacturer. The negative control shall be performed following the entire workflow of the on-site test and will therefore serve as a full process control to verify that the testing environment is not contaminated and that the end user can perform the test without introducing contaminants.

10.4.2.3 External positive controls performed by the end user

The manufacturer shall provide the end user with a procedure to perform a test that results in an expected positive result. This test may be performed with materials supplied by the manufacturer or commercially available materials specified by the manufacturer. The use of the external control material shall be validated by the manufacturer, including those supplied by the manufacturer or commercially available. The positive control shall be designed to verify that the end user can perform the test in the on-site testing environment and achieve a result in accordance with the validated performance characteristics of the testing system. If the test provides quantified results, the external positive control shall require that the system detect a result within the validated precision of the test system to pass. However, the result presented by the GUI to the end user is not required to be the quantified result. For example, the result presented may be “PASS” when the system calculates an accurately quantified control result.

10.5 No template control

For each batch of reagent manufactured, a NTC will be analysed to verify that there is no DNA contamination.

The NTC can either be a negative control as described in 10.4 or a blanks test for the PCR reagent.

A positive NTC may indicate contamination and requires additional verification of the test and investigation by the device manufacturer.

A NTC shall not be considered positive if it generates a PCR result that would not yield a positive result based on the device's result calculation algorithm.

10.6 Inhibition control

10.6.1 General

It is essential to assess the presence of PCR inhibitors in the device sample eluate.

The manufacturer shall include an inhibition control in the testing system. This inhibition control is either the target itself (see 10.6.2), a plasmid or an oligonucleotide (see 10.6.3).

10.6.2 Inhibition control is the target

For information on inhibition control is the target, refer to ISO/TS 12869:2019, 10.6.2.

10.6.3 Inhibition control is either a plasmid or an oligonucleotide

When the inhibition control is either a plasmid or an oligonucleotide, it will be co-amplified with the target. Table 4 provides the qualitative interpretation of the inhibition control results.

Dilution or an equivalent technique to decrease the concentration of inhibitor in the PCR reaction is required if quantification is impacted by inhibition.

Table 4 — 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	
Detected	No inhibition detected	*Legionella* spp. or *L. pneumophila* DNA is present.
Detected	Inhibition detected[a]	*Legionella* spp. or *L. pneumophila* DNA is present. If there is partial inhibition or competition, the sample shall be re-tested with less matrix input (e.g. dilution or decreased input volume [refer to 6.2.2]).
Not detected	No inhibition detected	There is no *Legionella* spp. or *L. pneumophila* DNA at the detection limit of the method.
Not detected	Inhibition detected[a]	If there is inhibition, the sample shall be re-tested with less matrix input (e.g. dilution or decreased input volume [refer to 6.2.2]).

[a] Detection of inhibition by the control can be identified through deviations in either slope and/or C_t value.

The device manufacturer shall fully validate the metrics for determination of inhibition detection.

Double stranded gene fragments, such as gBlocks, may also be validated and used as internal controls.

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 12869-2:2024;

b) the name of the manufacturer;

c) the product name;

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

e) all the information required to identify the consumable reagents used (e.g. lot number[s]);

f) sampling date and conditions;

g) the analysis date;

h) the filtered volume of the sample;

i) the results, expressed as described in Clause 8;

j) any details not included in this document that can affect the analytical results.