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

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

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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 documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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

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

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

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

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

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

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

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

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

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

Introduction

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

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

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

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

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

1 Scope

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

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

NOTE 1 For performance requirements, see Clause 9.

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

NOTE 2 For validation requirements, see 9.7.

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

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

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

2 Normative references

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

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

3 Terms, definitions, symbols and abbreviated terms

3.1 Terms and definitions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.6
C_t value
threshold cycle
number of PCR cycles (denaturation and amplification) required to replicate the DNA copies originally present in the sample, so that the concentration of DNA exceeds the detection limit

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

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

3.1.8
macrophage infectivity potentiator gene
***mip* gene**
gene present in *Legionella* spp. which is essential for the infection of the host (protozoa) and macrophages (humans)

Note 1 to entry: The unique base sequence of the *mip* gene of *L. pneumophila* can be used for the design of the primer and probe sequences for the specific qPCR detection of *L. pneumophila*.

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

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

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

3.1.10
recovery
efficiency of the DNA extraction method

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

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

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

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

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

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

3.1.14
working calibration solutions
L. pneumophila (WDCM 00107) DNA calibrated solutions, compared to the *L. pneumophila* DNA primary standard, used to establish the calibration curve

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

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

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

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

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

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

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

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

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

— dGTP: 2'-deoxyguanosine 5'-triphosphate

3.2 Symbols and abbreviated terms

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

4 Principle

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

— concentration of water samples by filtration;

— DNA extraction from the filter;

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

5 Sampling

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

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

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

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

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

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

6 General testing conditions

6.1 General

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

The principles to be applied are as follows:

— use of disposables compatible with PCR methods is preferred;

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

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

6.2 Staff

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

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

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

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

6.3 Premises

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

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

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

c) an area for PCR amplification.

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

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

6.4 Apparatus and consumables (excluding reagents)

6.4.1 Apparatus

Usual laboratory equipment, and in particular the following.

6.4.1.1 Biological safety cabinet (BSC II).

6.4.1.2 Centrifuge.

6.4.1.3 Heating block module.

6.4.1.4 Real-time thermocycler.

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

6.4.2 Consumables

All used consumables shall be free of DNA and DNAse.

EXAMPLE Filter funnels can be:

— delivered sterile;

— sterilized in an autoclave or oven;

— if made of metal, flamed prior to use.

6.4.3 Concentration

Membrane filters shall be made of polycarbonate or any other compound with a low capacity for adsorption of protein or DNA, with a nominal porosity of 0,45 µm or less. Do not use membrane filters containing cellulose or glass fibre.

6.4.4 Extraction and PCR (detection and quantification)

6.4.4.1 General

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

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

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

6.4.3.2 Micropipette

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

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

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

6.5 Reagents

6.5.1 General

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

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

Follow suppliers' recommendations for storage and handling of reagents.

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

6.5.2 PCR reagents

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

The reaction volumes handled during PCR tests are usually between 1 µl and 100 µl.

To increase PCR repeatability while decreasing the uncertainty associated with small volumes, sufficient volumes of reaction mixtures shall be prepared to enable at least 10 PCRs to be carried out.

Table 1 — Example of a typical PCR reaction mix

Component[a]	Comments
Dilution water	Diluent
PCR buffer solution	The composition varies greatly according to the supplier and various additives [bovine serum albumin, dimethyl sulfoxide (DMSO), surface active agents, etc.] appropriate for the activity or stability of the thermostable DNA polymerase used, can be added.
MgCl2	The final concentration MgCl2 depends on the dNTP, primers, probe, and target DNA concentrations. This shall be optimized:
dNTP	— dATP: 2'-deoxyadenosine 5'-triphosphate;
	— dTTP: 2'-deoxythymidine 5'-triphosphate;
	— dCTP: 2'-deoxycytidine 5'-triphosphate;
	— dGTP: 2'-deoxyguanosine 5'-triphosphate.
	A dTTP + dUTP (2'-deoxyuridine 5'-triphosphate) mix and a uracil-DNA N-glycosylase (UNG) enzyme can be used. This system is not mandatory for methods using a real-time detection system not requiring opening of tubes after amplification. Any equivalent system able to specifically destroy the amplicons from previous PCR, in the reaction mix, can be used.
Primers	See 7.3.2.2, 7.3.2.3, 7.3.2.5, 7.3.2.6.
Thermostable DNA polymerase	Use of hot-start Taq DNA polymerase is possible to avoid false-positive results.
Probes	See 7.3.2.4 and 7.3.2.7.
[a] Depending on their source, some of these components may previously be mixed in the PCR buffer solution.	

6.5.3 Other reagents

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

6.5.3.2 **TE buffer**, pH 8,0.

Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$)	Tris	10 mmol/l
Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2$)	EDTA	1 mmol/l

DNAse- and RNAse-free water

Dissolve the tris and EDTA in DNAse- and RNAse-free water and adjust with HCl to pH 8,0. For a 10-fold diluted TE buffer, dilute the solution with DNAse- and RNAse-free water.

6.6 Decontamination of equipment and premises

After accidental or non-accidental contamination, any recyclable equipment or material shall be treated by immersing in or soaking with, for example, a solution of bleach with 1,7 % volume fraction active chlorine or 1 % volume fraction hydrochloric acid or detergent.

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

6.7 Treatment and elimination of waste

Toxic and infectious waste shall be stored, used, and eliminated according to local regulations.

It is recommended that consumables contaminated by amplification products be discarded immediately.

7 Procedure

7.1 Concentration

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

7.2 DNA extraction

7.2.1 General

Extraction involves freeing the DNA by lysing the microorganisms, then (or at the same time) purifying the DNA while eliminating the other components as much as possible, particularly the PCR inhibitors. Check the recovery of the extract DNA (see 9.6).

7.2.2 Protocols

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

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

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

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

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

7.2.3 Stability of DNA extracts

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

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

7.3 DNA amplification by PCR

7.3.1 General

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

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

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

7.3.2 Target sequences, primers and probes

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

The specificity of the primers and probes shall be checked:

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

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

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

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

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