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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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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order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn.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*.

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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.

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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.

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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.

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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 °C ± 3 °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).

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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.

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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 Thermoblocs (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.

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6.5.2 PCR reagents

A PCR reaction mixture generally contains the components indicated in Table 1.

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 — Components used in a typical PCR reaction

Component [a]	Details
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
$MgCl_2$	Magnesium in its divalent cationic form is an essential co-factor of DNA polymerase activity. It forms a complex that is soluble with the dNTP. Its final concentration is thus dependent upon concentrations of dNTP, primers, probe, and target DNA. It shall be optimized
dNTP	Deoxyribonucleotide triphosphates used in synthesizing DNA by polymerase DNA:[b] dATP: 2'-deoxyadenosine 5'-triphosphate; dTTP: 2'-deoxythymidine 5'-triphosphate; dCTP: 2'-deoxycytidine 5'-triphosphate; dGTP: 2'-deoxyguanosine 5'-triphosphate
Primers	Oligonucleotides of determined size and sequence that determine the specific sequence to be amplified by PCR
Thermostable DNA polymerase	Enzyme or mix of enzymes used for *in-vitro* DNA polymerase reaction. NOTE Use of hot-start Taq DNA polymerase is possible to avoid false-positive results.
Probes	Oligonucleotides of determined size and sequence that hybridize on to a specific portion of the amplicon and which bear a fluorophore enabling the recognition of the fragment

[a] Depending on their source, some of these components may previously be mixed in the PCR buffer solution (ready-to-use PCR master mix products including the components, except primers and probe are available).

[b] A dTTP + dUTP (2'-deoxyuridine 5'-triphosphate) mix and a uracil-DNA *N*-glycosylase (UNG) enzyme can be used. This system is not mandatory for methods using a real-time detection system not requiring opening of tubes after amplification. Any equivalent system able to specifically destroy the amplicons from previous PCR, in the reaction mix, can be used.

6.5.3 Other reagents

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

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.

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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 °C ± 3 °C and analysed within 24 h of preparation. Any longer storage at this temperature requires validation.

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

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