



SLOVENSKI STANDARD SIST-TS ISO/TS 12869:2019

01-oktober-2019

Nadomešča:

SIST-TS ISO/TS 12869:2013

Kakovost vode - Ugotavljanje prisotnosti in števila Legionella spp. in/ali Legionella pneumophila s koncentriranjem in pomnoževanjem genov s kvantitativno verižno reakcijo s polimerazo (qPCR)

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

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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)

Ta slovenski standard je istoveten z: ISO/TS 12869:2019

ICS:

07.100.20	Mikrobiologija vode	Microbiology of water
13.060.70	Preiskava bioloških lastnosti vode	Examination of biological properties of water

SIST-TS ISO/TS 12869:2019

en,fr,de

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TECHNICAL
SPECIFICATIONISO/TS
12869Second edition
2019-04

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

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et/ou *Legionella pneumophila* par concentration et amplification
génique par réaction de polymérisation en chaîne quantitative (qPCR)*

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ISO/TS 12869:2019(E)

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Published in Switzerland

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Foreword

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

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

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.

ISO/TS 12869:2019(E)**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).

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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. 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.

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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 C_q 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)

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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](#)).

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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.

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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.
MgCl ₂	The final concentration MgCl ₂ 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.