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Technical Specification

ISO/DTS 16099

Water quality — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms and viruses — General requirements, quality assurance and validation

Qualité de l'eau — Réaction de polymérisation en chaîne (PCR) pour la détection et la quantification des microorganismes et des virus — Exigences générales, assurance de la qualité et validation

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Foreword

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

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

PCR-based methods are developed for the detection and/or quantification of, for example, pathogenic bacteria, for rapid and reliable outcomes as an alternative to culture-based methods. For example, for the screening on the presence of *Legionella* or faecal-related microorganisms in water, see References [26], [29], [43], [49] and [56] for further information.

Performing nucleic acid quantification assays to a high standard of analytical quality can be challenging. For example, it is well known that impure or degraded nucleic acid extracts can affect the accuracy of quantification. Similarly, a poorly designed quantitative polymerase chain reaction (qPCR) assay with poor amplification efficiency and poor primer specificity will impact the quantification accuracy of nucleic acid targets.

In addition, aspects such as the water matrix and standard curves can have a significant influence on the accuracy of quantitative measurements of nucleic acid targets. Therefore, it is important to improve the reliability of data by setting general requirements for PCR-based methods.

Water quality — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms and viruses — General requirements, quality assurance and validation

1 Scope

This document specifies the general requirements for the in vitro amplification of nucleic acid sequences (DNA or RNA). This includes polymerase chain reaction (PCR)-based methods like quantitative PCR, qualitative PCR, reverse transcription-PCR and digital PCR.

The minimum requirements laid down in this document are intended to ensure that comparable and reproducible results are obtained in different organizations. It covers quality assurance aspects to be considered when working with PCR-based methods in a laboratory as well as validation and verification.

In addition to laboratory PCR-based methods, this document is also applicable to on-site PCR-based methods.

This document is applicable to PCR-based methods used for the analysis of microorganisms and viruses in different water matrices, including but not limited to:

— drinking water;

— groundwater;

— pool water;

— process water;

— surface water;

— wastewater.

This document is applicable to the detection and quantification of nucleic acids (DNA or RNA) of microorganisms by PCR-based methods in water such as bacteria, yeasts, fungi but also parasites such as *Cryptosporidium, Giardia,* amoebas and multicellular organisms. In addition, this document is applicable to the detection and quantification of nucleic acids from viruses in water by PCR-based methods.

NOTE In the context of this document, viruses are considered to be microorganisms. Clauses in this document can also specifically apply to viruses and not to other types of microorganisms. In these clauses, viruses are mentioned separately.

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*

ISO 20836, *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers*

3 Terms and definitions

For the purposes of this document, the following terms and definitions 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
5′-3′-exonuclease activity
ability of *deoxyribonucleic acid (DNA) polymerase* (3.18) to cleave nucleotides in the 5′-3′-direction

Note 1 to entry: When a fluorescent *probe* (3.50) is used for the detection of amplification, the 5'-3'-exonuclease activity of DNA polymerase allows the probe to hydrolyse and emit fluorescence.

3.2
absolute quantification digital polymerase chain reaction
absolute quantification by dPCR
procedure involving PCR amplification and target copy quantification which does not require a standard curve to determine the concentration of a target *nucleic acid* (3.31) in a sample

[SOURCE: ISO 22174:2024, 3.7.9]

3.3
absolute quantification by real-time polymerase chain reaction
absolute quantification by real-time PCR
procedure involving PCR amplification to determine the concentration of a target *nucleic acid* (3.31) in a sample by comparison with a standard curve, derived from standards containing a defined amount of target

[SOURCE: ISO 22174:2024, 3.6.4]

3.4
aliquot
portion of a quantity of liquids which has been divided into separate parts at the same time under identical conditions

3.5
amplification control
nucleic acid (3.31) added in a defined amount or copy number which serves as a control for amplification

Note 1 to entry: This *nucleic acid sequence* (3.34) can be endogenous [naturally present in the tested *matrix* (3.25)] or exogeneous (naturally absent in the tested matrix).

Note 2 to entry: The amplification control can either be an internal or external control. With the internal amplification control, the control is added to each polymerase chain reaction (PCR); this requires the use of a *multiplex PCR* (3.26). The external amplification control is added to each aliquot of the extracted nucleic acid serving as a control for amplification in a separate reaction.

Note 3 to entry: An exogenous internal amplification control can be homologous (amplified using the same *primers* (3.47) as used for amplification of the target) or heterologous (amplified using different primers than those used for amplification of the target). A homologous internal amplification control amplicon shall be distinguishable from the microbial target amplicon (e.g. by size or by insertion of a different probe-binding sequence).

3.6
annealing
pairing of complementary single strands of *nucleic acids* (3.31) to form a double-stranded molecule

Note 1 to entry: Lowering the temperature of the PCR reaction allows *primers* (3.47) and *probes* (3.50) to pair with a complementary single-stranded nucleic acid to form a double-stranded molecule.

[SOURCE: ISO 22174:2024, 3.4.13, modified — Note 1 to entry has been added.]

3.7
background fluorescence
background
intrinsic level of fluorescence resulting from the reagents, consumables and instruments used

[SOURCE: ISO 22174:2024, 3.4.7]

3.8
complementary DNA
cDNA
single-stranded *deoxyribonucleic acid (DNA)* (3.12), complementary to a given *ribonucleic acid (RNA)* (3.63) and synthesised in the presence of reverse transcriptase to serve as a template for *DNA amplification* (3.17)

[SOURCE: ISO 20395:2019, 3.5]

3.9
cross-contamination
unintended transfer of *nucleic acids* (3.31) [*deoxyribonucleic acid (DNA)* (3.12) or *ribonucleic acid (RNA)* (3.63)]

EXAMPLE Cross-contamination can occur between samples during PCR preparation.

3.10
decontamination
procedure to remove or reduce *nucleic acids* (3.31) and/or nucleases from materials and surfaces

3.11
denaturation
process which results in the separation of the double-stranded *nucleic acid* (3.31) into single-stranded nucleic acids

[SOURCE: ISO 22174:2024, 3.4.11]

3.12
deoxyribonucleic acid
DNA
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2024, 3.1.6]

3.13
deoxyribonuclease
DNase
enzyme which degrades *deoxyribonucleic acid (DNA)* (3.12)

[SOURCE: ISO 22174:2024, 3.1.7]

3.14
deoxyribonucleoside triphosphate
dNTP
solution containing deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and/or deoxyuridine triphosphate (dUTP)

[SOURCE: ISO 22174:2024, 3.4.2]

3.15
detection of polymerase chain reaction product
detection of PCR product
detection of amplicon
process which signals the presence of a PCR product

[SOURCE: ISO 22174:2024, 3.1.15, modified — “an amplicon” has been replaced by “a PCR product”.]

3.16
digital polymerase chain reaction
digital PCR
dPCR
procedure in which *nucleic acid* (3.31) templates are randomly and independently distributed across multiple *partitions* (3.37) of nominally equivalent volume, such that some partitions contain template and others do not, followed by PCR amplification of target sequences and detection of specific PCR products, providing a count of the number of partitions with a positive and negative signal for the target template

Note 1 to entry: dPCR can also provide the qualitative results “detected” or “not detected”.

Note 2 to entry: In certain instances, whole cells or organisms can be partitioned and lysis is performed in the individual partitions to allow amplification of target templates.

[SOURCE: ISO 22174:2024, 3.7.1, modified — “amplicons” has been replaced by “PCR products”.]

3.17
deoxyribonucleic acid amplification
DNA amplification
multiplication of *nucleic acid* (3.31) through polymerase chain reaction (PCR) resulting in the amplified PCR product

Note 1 to entry: The filling-in of the single-stranded *DNA* (3.12) by *DNA polymerase* (3.18) to double-stranded DNA, starting from the *primer* (3.47) binding site, is also called elongation.

3.18
deoxyribonucleic acid polymerase
DNA polymerase
thermostable enzyme which catalyses *DNA* (3.12) synthesis

Note 1 to entry: DNA polymerase can also cleave a hybridized *nucleic acid* (3.31) molecule using its *5′-3′-exonuclease activity* (3.1). It is dependent on the type of enzyme and can be present in, for example, Taq-, Tth- and Tfl-polymerase.

Note 2 to entry: DNA synthesis is catalysed by reading the existing DNA sequence, then adding nucleotides to the newly forming strand of DNA that are complementary to the original strand. By using a forward and a reverse *primer* (3.47), DNA polymerases will duplicate both strands of the original source DNA resulting in two identical DNA molecules.

[SOURCE: ISO 22174:2024, 3.4.1, modified — Note 2 to entry has been added.]

3.19
eluate
solution obtained through *nucleic acid extraction* (3.32) and purification (3.33) that contains the *nucleic acids* (3.31) of a sample and that is to be used for PCR

3.20
hybridization
specific binding of complementary *nucleic acid sequences* (3.34) under suitable reaction conditions

[SOURCE: ISO 22174:2024, 3.4.12]

3.21
intercalating dye
double-stranded DNA binding dye that emits fluorescence when bound to double-stranded DNA, that gets also inserted into double-stranded DNA during the process of PCR amplification, increasing the intensity of the fluorescence with the increase of double-stranded DNA

3.22
internal process control
control used for the quality assessment of the entire protocol, which is therefore added to the investigated sample material to undergo the same procedure as the naturally present microorganism or genetic material

Note 1 to entry: Due to its mode of action, an internal control shall be selected which assumingly is naturally absent in the tested *matrix* (3.25).

Note 2 to entry: For optimal use of the internal process control, it is recommended to use a control that behaves the same way in all parts of the PCR-based method, e.g. a virus as a control for a virus PCR-based method and, in case of bacteria, those that exhibit similar extraction behaviour (e.g. gram-positive or gram-negative).

[SOURCE: ISO 22174:2024, 3.5.3, modified — "target microorganism" has been replaced by "naturally present microorganism or genetic material" and Note 2 to entry has been added.]

3.23
limit of blank
LoB
<digital polymerase chain reaction> highest number of *partitions* (3.37) appearing positive, with more than 95 % probability, when testing samples in the absence of the target *nucleic acid sequence* (3.34) of the *target organism* (3.66), which determines the target sequence specific "false positive" limit

Note 1 to entry: The LoB should be determined, as a minimum, from replicates of the negative *amplification control* (3.5) (amplification LoB) (e.g. water, elution buffer) and negative samples containing the *matrix* (3.25) (full method LoB). The number of negative control replicates and negative samples should be justified by the user laboratory and should be consistent with the validation tests performed by the developer.

[SOURCE: ISO 22174:2024, 3.7.7, modified — "pathogen" has been replaced by "target organism".]

3.24
master mix
mixture of reagents needed for *nucleic acid* (3.31) amplification except for the target *nucleic acid sequence* (3.34)

Note 1 to entry: A for reverse transcription (RT)-master mix is a type of master mix that contains a mixture of reagents needed for reverse transcription, at least consisting of a *reverse transcriptase* (3.58), a *primer* (3.47), *deoxynucleotide triphosphates (dNTPs)* (3.14), *ribonuclease* (3.61) inhibitor and polymerase chain reaction-grade water (*PCR-grade water)* (3.39).

Note 2 to entry: A PCR-master mix is a type of master mix that contains a mixture of reagents needed for *DNA amplification* (3.17), at least consisting of a *deoxyribonucleic acid (DNA) polymerase* (3.18), primers, dNTPs and PCR-grade water.

[SOURCE: ISO 22174:2024, 3.4.4, modified — Notes 1 and 2 to entry have been added.]

3.25
matrix
all the components of the sample

[SOURCE: ISO 22174:2024, 3.1.5]

3.26
multiplex polymerase chain reaction
multiplex PCR
PCR allowing the detection of multiple targets simultaneously within a single reaction tube, where more *primer* (3.47) pairs [and *probes* (3.50)] are used within one *master mix* (3.24)

[SOURCE: ISO 22174:2024, 3.1.20]

3.27
negative cluster
set of negative results from *partitions* (3.37) that contained the reaction mix, without the target sequence, representing the negative partitions

[SOURCE: ISO 22174:2024, 3.7.3]

3.28
negative extraction control
extraction blank
control carried through all steps of the *nucleic acid extraction* (3.32) procedure in the absence of a sample

[SOURCE: ISO 22174:2024, 3.5.4, modified — "test" has been deleted.]

3.29
negative polymerase chain reaction control
negative PCR control
no-template control
NTC
PCR control made with water (or other PCR-inert substrate such as grinding or elution buffer) free of target *nucleic acid* (3.31) and PCR inhibitors

[SOURCE: ISO 22174:2024, 3.5.8]

3.30
negative process control
target free sample which is run through all stages of the analytical process

Note 1 to entry: The process can include sample preparation, enrichment, *nucleic acid extraction* (3.32) and target amplification.

[SOURCE: ISO 22174:2024, 3.5.1]

3.31
nucleic acid
polymer of deoxyribonucleotides or ribonucleotides

[SOURCE: ISO 22174:2024, 3.1.11]

3.32
nucleic acid extraction
sample treatment for the release of *nucleic acids* (3.31)

[SOURCE: ISO 22174:2024, 3.2.1]

3.33
nucleic acid purification
method to reduce the amount of polymerase chain reaction (PCR) inhibitors in the *eluate* (3.19)

[SOURCE: ISO 22174:2024, 3.2.2]

3.34
nucleic acid sequence
order of nucleotides which are specific for a certain target that serves as a template for amplification during of polymerase chain reaction (PCR)

3.35
oligonucleotide
short synthetic single-stranded *nucleic acid* (3.31) fragment (usually between 10 to 40 nucleotides in length)

3.36
on-site polymerase chain reaction
on-site PCR
PCR-based method carried out near, or on the same location outside the laboratory, where the water sampling and *nucleic acid purification* (3.33) has been carried out

Note 1 to entry: For on-site PCR, the conditions in which the reactions are carried out can differ greatly. Therefore, it is recommended that the entire method is suitable for the potentially differing conditions.

3.37
partition
droplet or chamber of nominally equivalent volume into which *digital PCR (dPCR)* (3.16) mix of reagents and template is randomly distributed and then amplified by PCR

[SOURCE: ISO 20395:2019, 3.22]