International Standard

ISO 22174

Second edition
2024-08

# Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms — General requirements and definitions

*Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la recherche et la quantification de micro-organismes — Exigences générales et définitions*



Reference number
ISO 22174:2024(en)



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Published in Switzerland

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# 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 TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces ISO 22174:2005, ISO 20837:2006, ISO 20838:2006 and ISO 22119:2011, which have been technically revised.

The main changes are as follows:

— inclusion of requirements for the implementation of digital PCR;

— inclusion of requirements for laboratory flows monitoring including environmental monitoring for PCR;

— extension of 12.2.2 control reaction with descriptions of the different controls;

— change of 12.3 to include quantitative evaluation;

— inclusion of Clause 14 on validation and verification.

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.

# Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms — General requirements and definitions

## 1 Scope

This document specifies the general requirements for the *in vitro* amplification of nucleic acid sequences (DNA or RNA).

This document is applicable to the testing for microorganisms and viruses from the food chain using the polymerase chain reaction (PCR). This document, or parts of it, is applicable to other fields of PCR diagnostics based on a case-by-case evaluation.

The minimum requirements laid down in this document are intended to ensure that comparable and reproducible results are obtained in different laboratories.

This document has been established for microorganisms from the food chain and is applicable to:

— products intended for human consumption;

— products for feeding animals;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

## 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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of the food chain — General requirements and guidance for microbiological examinations*

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 General terms

**3.1.1**
**laboratory sample**
sample as prepared for sending to the laboratory and intended for inspection or testing

[SOURCE: ISO 7002:1986, A.19]

**3.1.2**
**test sample**
sample prepared from the *laboratory sample* (3.1.1) according to the procedure specified in the method of test and from which *test portions* (3.1.3) are taken

**3.1.3**
**test portion**
measured (volume or mass) representative sample taken from the *laboratory sample* (3.1.1)

[SOURCE: ISO 6887-1:2017, 3.5, modified — "for use in the preparation of the initial suspension" and Note 1 to entry deleted.]

**3.1.4**
**reference material**
material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

Note 1 to entry: Reference material producers fulfilling the requirements of ISO 17034 are considered to be competent.

[SOURCE: ISO Guide 30:2015, 2.1.1, modified — Notes to entry deleted and a new Note 1 to entry added.]

**3.1.5**
**matrix**
all the components of the sample

[SOURCE: ISO 16140-1:2016, 2.38, modified — "(product)" deleted in the term.]

**3.1.6**
**deoxyribonucleic acid**
**DNA**
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

**3.1.7**
**deoxyribonuclease**
**DNase**
enzyme which degrades *deoxyribonucleic acid (DNA)* (3.1.6)

**3.1.8**
**amplicon**
*deoxyribonucleic acid (DNA)* (3.1.6) amplified by *polymerase chain reaction (PCR)* (3.1.17)

**3.1.9**
**ribonucleic acid**
**RNA**
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

**3.1.10**
**ribonuclease**
**RNase**
enzyme which degrades *ribonucleic acid (RNA)* (3.1.9)

**3.1.11**
**nucleic acid**
polymer of deoxyribonucleotides or ribonucleotides

**3.1.12**
**target nucleic acid sequence**
nucleic acid sequence selected for amplification

**3.1.13**
**endogenous sequence**
nucleic acid sequence naturally present in the tested *matrix* (3.1.5)

**3.1.14**
**exogenous sequence**
nucleic acid sequence naturally absent in the tested *matrix* (3.1.5)

**3.1.15**
**detection of polymerase chain reaction product**
**detection of amplicon**
process which signals the presence of an *amplicon* (3.1.8)

**3.1.16**
**confirmation of polymerase chain reaction product**
**confirmation of amplicon**
process which demonstrates that the *amplicon* (3.1.8) originates from the *target nucleic acid sequence* (3.1.12)

**3.1.17**
**polymerase chain reaction**
**PCR**
enzymatic procedure that allows in vitro amplification of *deoxyribonucleic acid (DNA)* (3.1.6)

**3.1.18**
**endpoint polymerase chain reaction**
**endpoint PCR**
procedure using PCR amplification followed by separate detection of *amplicons* (3.1.8) after the completion of the PCR

**3.1.19**
**real-time polymerase chain reaction**
**real-time PCR**
procedure which combines PCR amplification with the detection and/or quantification of specific *amplicons* (3.1.8) during the amplification process

**3.1.20**
**multiplex polymerase chain reaction**
**multiplex PCR**
*PCR* (3.1.17) allowing the detection of multiple targets simultaneously within a single reaction tube, where more primer pairs (and probes) are used within one *master mix* (3.4.4)

## 3.2 Terms related to the extraction and purification of DNA/RNA

**3.2.1**
**nucleic acid extraction**
sample treatment for the release of *nucleic acids* (3.1.11)

**3.2.2**
**nucleic acid purification**
method to reduce the amount of *polymerase chain reaction (PCR)* (3.1.17) inhibitors in the eluate

## 3.3 Terms related to reverse transcription of RNA to DNA

**3.3.1**
**reverse transcriptase**
enzyme which catalyses the *reverse transcription* (3.3.2) of *ribonucleic acid (RNA)* (3.1.9) to a complementary single-stranded deoxyribonucleic acid (cDNA)

**3.3.2**
**reverse transcription**
**RT**
synthesis of complementary single-stranded deoxyribonucleic acid (cDNA) from a ribonucleic acid (RNA) template using a *reverse transcriptase* (3.3.1)

**3.3.3**
**reverse transcription-polymerase chain reaction**
**RT-PCR**
method consisting of two reactions, a *reverse transcription (RT)* (3.3.2) of *ribonucleic acid (RNA)* (3.1.9) to single-stranded complementary deoxyribonucleic acid (cDNA), followed by a *PCR* (3.1.17)

Note 1 to entry: One-step RT-PCR is performed in a single tube.

Note 2 to entry: Two-step RT-PCR can either be performed sequentially in a single tube or in two different tubes.

## 3.4 Terms related to DNA amplification by PCR/RT-PCR

**3.4.1**
**deoxyribonucleic acid polymerase**
**DNA polymerase**
thermostable enzyme which catalyses *DNA* (3.1.6) synthesis

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

**3.4.2**
**deoxyribonucleoside triphosphate**
**dNTP**
solution containing deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and/or deoxyuridine triphosphate (dUTP)

**3.4.3**
**thermal cycler**
automatic device that performs defined heating and cooling cycles usable for *polymerase chain reaction (PCR)* (3.1.17) or *real-time PCR* (3.1.19) or *digital PCR* (3.7.1)

Note 1 to entry: The thermal cycler can be a block-based or (individual) reaction-chamber-based thermal cycler.

[SOURCE: ISO 20836:2021, 3.2.1, modified — “or digital PCR” added.]

**3.4.4**
**master mix**
mixture of reagents needed for nucleic acid amplification except for the *target nucleic acid sequence* (3.1.12)

[SOURCE: ISO 17822:2020, 3.27, modified — “nucleic acid sequence” replaced “DNA and the controls”.]

**3.4.5**
**primer**
oligonucleotide of defined length and sequence complementary to a segment of the *target nucleic acid sequence* (3.1.12), used to signal the starting point for deoxyribonucleic acid (DNA) polymerase to extend the new DNA strand

**3.4.6**
**fluorescent probe**
oligonucleotide of defined sequence coupled with one or more fluorescent molecules

Note 1 to entry: Any system emitting a fluorescence signal after specific hybridization to the *target nucleic acid sequence* (3.1.12) which can be detected by the specific equipment can be used as a fluorescent probe.

**3.4.7**
**background fluorescence**
background
intrinsic level of fluorescence resulting from the reagents, consumables and instruments used

**3.4.8**
**molecular beacon**
fluorescent probe consisting of three different parts: a central part complementary to the *target nucleic acid sequence* (3.1.12), plus a 5′-part and a 3′-part which are complementary, and where the reporter is attached to one arm of the molecule, while the end of the other arm carries the quencher

**3.4.9**
**hybridization probe**
system of two fluorescent probes coupled with one fluorescent molecule each, where one molecule serves as fluorescence resonance energy transfer (FRET) donor and the other serves as FRET acceptor

**3.4.10**
**hydrolysis probe**
fluorescent probe coupled with a fluorophore and quencher which are sterically separated by the 5′-3′-exonuclease activity of the enzyme during the amplification process

**3.4.11**
**denaturation**
process which results in the separation of the double-stranded nucleic acid into single-stranded nucleic acids

**3.4.12**
**hybridization**
specific binding of complementary nucleic acid sequences under suitable reaction conditions

**3.4.13**
**annealing**
pairing of complementary single strands of nucleic acids to form a double-stranded molecule

**3.4.14**
**hot-start polymerase chain reaction**
**hot-start PCR**
activation of thermostable deoxyribonucleic acid (DNA) polymerase by an initial heating step to avoid non-specific amplification

## 3.5 Terms related to controls

**3.5.1**
**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 and target amplification.

**3.5.2**
**positive process control**
sample, spiked with a microorganism, treated in the same way as the *test samples* (3.1.2) to monitor the entire process of the polymerase-chain-reaction-based method

**3.5.3**
**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 target microorganism

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.

**3.5.4**
**negative extraction control**
extraction blank
control carried through all steps of the nucleic acid extraction procedure in the absence of a *test sample* (3.1.2)

**3.5.5**
**internal amplification control**
*nucleic acid* (3.1.11) added to each reaction in a defined amount or copy number which serves as an internal control for amplification

Note 1 to entry: This nucleic acid sequence can be endogenous (naturally present in the tested matrix) or exogenous (naturally absent in the tested matrix).

Note 2 to entry: An exogenous internal amplification control can be homologous (amplified using the same primers 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).

**3.5.6**
**external amplification control**
control nucleic acid added to an aliquot of the extracted nucleic acid in a defined amount or copy number serving as a control for amplification in a separate reaction

Note 1 to entry: It is strongly recommended that the external amplification control amplicon is distinguishable from the target amplicon (e.g. by insertion of a restriction enzyme target sequence).

**3.5.7**
**positive polymerase chain reaction control**
**positive PCR control**
reaction containing the target nucleic acid in a defined amount or copy number

**3.5.8**
**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 and PCR inhibitors

## 3.6 Terms related to qPCR

**3.6.1**
**quantitative polymerase chain reaction**
**qPCR**
method allowing the quantification in a nucleic acid template of the number of a specific nucleic acid sequence using specific oligonucleotides

**3.6.2**
**quantification cycle**
$C_q$
<real-time polymerase chain reaction> cycle at which the fluorescence signal can be distinguished from the *background fluorescence* (3.4.7) entering into the exponential phase of target amplification

Note 1 to entry: Quantification cycle is a generic term which includes cycle threshold ($C_t$), crossing point ($C_p$), take off point (TOP) and all other instrument specific terms referring to the fractional cycle used to detect or quantify the target in the real-time PCR assay.

Note 2 to entry: The quantification cycle is based either on a threshold applied to all samples or on a regression analysis of the signal, for each sample.

[SOURCE: ISO 20395:2019, 3.8, modified — The definition has been revised. “real-time PCR” replaced “qPCR” in the domain and Note 1 to entry.]

**3.6.3**
**relative quantification by real-time polymerase chain reaction**
**relative quantification by real-time PCR**
procedure involving PCR amplification to determine the levels of changes of a target nucleic acid relative to the levels of a reference nucleic acid of known concentration, measured within the same or in separate PCR reactions

**3.6.4**
**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 in a sample by comparison with a standard curve, derived from standards containing a defined amount of target

**3.6.5**
**passive reference**
<real-time polymerase chain reaction> fluorescent molecules present in the reaction mix used to normalize the signal

## 3.7 Terms related to dPCR

**3.7.1**
**digital polymerase chain reaction**
**digital PCR**
**dPCR**
procedure in which nucleic acid templates are randomly and independently distributed across multiple *partitions* (3.7.2) 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 *amplicons* (3.1.8), 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 20395:2019, 3.10, modified — “are randomly and independently” added before “distributed”. Original Notes 1 and 2 to entry deleted and new Notes to entry added.]

**3.7.2**
**partition**
droplets or chambers of nominally equivalent volume into which *digital polymerase chain reaction* (*dPCR)* (3.7.1) mix of reagents and template is randomly distributed and then amplified by PCR

[SOURCE: ISO 20395:2019, 3.22]

**3.7.3**
**negative cluster**
set of negative results from partitions that contained the reaction mix, without the target sequence, representing the negative partitions

**3.7.4**
**positive cluster**
set of positive results from partitions that contained the reaction mix, with amplification of the target sequence, representing the positive partitions

**3.7.5**
**separability**
distance between the *negative cluster* (3.7.3) and the *positive cluster* (3.7.4) in the analysis diagram (1D Dot Plot) generated after reading the partition signals

Note 1 to entry: Separability can vary depending on the quality of the sample and the amount of target. Therefore, it is important to pay attention to the separability of these two clusters when a weak positive sample is detected.

**3.7.6**
**analysis threshold**
<digital polymerase chain reaction> limit of separation between the *negative cluster* (3.7.3) and the *positive cluster* (3.7.4)

Note 1 to entry: The threshold limit can be set automatically by the analysis software or manually by the operator

**3.7.7**
**limit of blank**
**LoB**
<digital polymerase chain reaction> highest number of partitions appearing positive, with more than 95 % probability, when testing samples in the absence of the target nucleic acid sequence of the pathogen, 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 (amplification LoB) (e.g. water, elution buffer) and negative samples containing the matrix (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.

**3.7.8**
**passive reference**
<digital polymerase chain reaction> fluorescent molecule in the reaction medium allowing to count the *partitions* (3.7.2) containing the reaction mixture

**3.7.9**
**absolute quantification by 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 in a sample



# 4 Principle

## 4.1 General

The procedure comprises the following consecutive steps:

a) preliminary microbial enrichment of the food-borne microorganism or concentration of the virus from the laboratory sample, if required (see Clause 5);

b) nucleic acid extraction and purification, if required (see Clause 6);

c) amplification of the target nucleic acid sequence by PCR or RT-PCR using specific primers with the addition of fluorescent probes or DNA double strand binding dyes in the case of real-time and digital format (see Clause 7);

d) detection and confirmation of the specific amplicons on electrophoresis gel (endpoint PCR) or by monitoring the fluorescence signal with an optical detection system (real-time or digital format) (see Clause 8);

e) data analysis;

f) evaluation.

### 4.2 Laboratory sample

Any material from the food chain is suitable as a laboratory sample, provided it has been established by the use of a relevant control (see Table 1) that the nucleic acid solution prepared from the test portion does not completely inhibit the PCR.

### 4.3 Sampling, transport and storage

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. Further details of sampling certain products are given in specific standards that are listed in ISO 7218.

If there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receives a sample that is representative. Samples transported to the laboratory are kept under conditions which will minimize any alteration in the number of microorganisms present. The sample should not have been damaged or changed during transport or storage.

The recommended temperatures for samples during transport and storage are described in ISO 7218.

### 4.4 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned. Follow the procedures specified in the ISO 6887 series. The preparation of the test sample for the detection of viruses can be different and is specified in, for example, the ISO 15216 series for some products. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

## 5 Microbial enrichment and virus concentration

### 5.1 Microbial enrichment

Microbial enrichment may start directly from the test portion. This can be done by stimulating growth of the target microorganism by culturing the test portion in selective or non-selective liquid nutrient media. Filtration and/or concentration such as immunomagnetic separation can be also used. Specific antimicrobial compounds can be used to inhibit the growth of non-target microorganisms.

### 5.2 Virus concentration

The foodstuffs and food surfaces are often highly complex matrices and the target viruses can be present at low concentrations. It is therefore necessary to carry out matrix-specific virus extraction and/or concentration in order to provide a substrate for subsequent common parts of the process. The method depends upon the matrix, some of which are specified in the ISO 15216 series.

# 6 Nucleic acid preparation

## 6.1 General

The purpose of the nucleic acid preparation step is to obtain a nucleic acid solution from the laboratory sample that does not significantly inhibit the PCR. The microbial cells in the laboratory sample or enriched/concentrated culture (see Clause 5) are lysed to release their nucleic acid (DNA and/or RNA). If required, a concentration stage (e.g. centrifugation or filtration) is performed prior to lysis and/or a purification step is performed following lysis. The nucleic acid solution obtained from the extraction step should contain a sufficient amount of target nucleic acid of suitable quality.

The resulting nucleic acid solution should contain as few as possible substances with detectable PCR inhibition or fluorescence interference.

NOTE 1 Fluorescence is often derived from coloured reaction vessels and some ingredients of enrichment broths.

Depending on the sample received it can be necessary to pre-treat or dilute the test portion, i.e. some products can contain compounds that inhibit PCR. If PCR inhibition is identified (by the use of a control reaction, see 12.3), the enriched test portion or the lysate can be diluted prior to the PCR step.

NOTE 2 The method of extraction can have a great influence on nucleic acids degradation and/or yield and/or amplification efficiency. The most suitable extraction method strongly depends on the characteristics of the test portion.

## 6.2 Prevention of amplification of DNA from dead cells

Some test portions can contain high amounts of DNA originating from dead cells of the target organism (e.g. highly processed samples, environmental samples collected after disinfection or phage treated samples). These dead cells, if present in high enough concentrations, can lead to PCR positive results, even though no living organisms are present. To remove or inactivate DNA from dead cells prior to nucleic acid preparation, a pre-treatment may be used if viable target organisms are not significantly affected by the process.

NOTE 1 Dead cells in this context are cells which have a damaged cell wall which allows certain DNA-binding chemicals to penetrate into the cell and prevent subsequent PCR amplification.

NOTE 2 The yield of DNA removal from dead cells can be affected by different factors. The protocol performance can be enhanced if some critical factors are considered.[17]

## 6.3 Nucleic acid extraction, release and purification

Several DNA or RNA extraction principles may be used. For example, the following methods can be used:

a) Protein digestion: Break down proteins in the cell extract with proteases (e.g. Proteinase K) and RNA with ribonucleases if relevant. Precipitate the resulting peptides then purify the nucleic acid solution and concentrate further by ethanol precipitation in the presence of monovalent cations.

b) Thermal lysis: Nucleic acids can also be released by thermal cell disruption (e.g. by boiling for 10 min). After the thermal cell disruption is complete, allow the sample to cool. Centrifuge the sample and use the supernatant for PCR analysis. Before the thermal cell disruption and to facilitate cell disruption, enzymatic treatment can be applied (e.g. lysozyme, for use with Gram-positive bacteria) followed by a protease/proteinase incubation.

c) Other methods such as vigorous agitation with beads can be required when the organism has a particularly tough cell wall (e.g. *Mycobacterium* spp.).

NOTE Commercial kits for nucleic acid extraction, release and purification are available on the market.

## 6.4 Nucleic acid quality and quantity

The quality and yield of nucleic acid extracted by a given method on a given matrix should be both repeatable and reproducible in terms of amplification by PCR, provided there is sufficient nucleic acid in the matrix. In

particular, the method used shall allow the recovery of nucleic acid fragments with an average size equal to or greater than the amplicons under investigation.

For some sample preparation methods and when the nucleic acids released are not stable, it is necessary to use the nucleic acid solution immediately after the preparation. It is recommended to use nucleic extraction methods which inactivate or remove DNases and RNases. The pH of the lysate should be suitable for DNA and RNA stability.

In general, repeated freezing and thawing of nucleic acid solutions should be avoided. Use low binding plasticware to store low copy numbers of nucleic acids, especially RNA.

The concentration of the nucleic acids isolated can be estimated by spectrophotometric or fluorometric methods or by gel electrophoresis.

The purity of the nucleic acids isolated can be estimated by spectrophotometric methods or by gel electrophoresis.

## 7 PCR amplification

A specific nucleic acid sequence is amplified using PCR (multiple nucleic acid sequences can also be targeted simultaneously in a multiplex PCR format). The reaction is a cyclic process consisting of three stages:

a) denaturation of the double-stranded nucleic acid (dsDNA);

b) annealing of the primers (and probe) to the complementary target sequence;

c) extension of the attached primers by means of a thermostable DNA polymerase.

To reduce non-specific amplification, a hot-start master mix can be used together with an initial heating step to perform a hot-start PCR.

RNA can be detected using RT-PCR, where the target is first transcribed into a complementary DNA (cDNA) by reverse transcription with a reverse transcriptase. In the real-time PCR format, the fluorescence signal is monitored in each cycle during the annealing stage or the extension stage depending on the probe used.

In the case of dPCR, reaction mix containing sample nucleic acid is randomly and independently distributed into discrete partitions of equivalent volume such that some partitions contain no nucleic acid template and others contain one or more template copies. The partitions are then thermally cycled to end-point.

NOTE 1 Following denaturation of double-stranded DNA, two oligonucleotide primers anneal (hybridize) to the target DNA segment to be amplified. The primers are directed opposite to each other regarding their orientation to the target sequence.

NOTE 2 Double-stranded regions are formed as a result of specific base-pairing between the primers and the target sequence flanking the DNA segment to be amplified and serve as start positions for DNA synthesis by means of a heat-stable DNA polymerase.

NOTE 3 The repeated process of heat denaturation, primer annealing and DNA synthesis (cycles) results in the near exponential amplification of the DNA segment flanked by the primers.

NOTE 4 The annealing and elongation stage can be combined into one stage which results in a two-stage PCR cycle.

NOTE 5 In certain instances, whole cells or organisms can be partitioned and lysis is performed in the individual partitions to allow amplification of target templates.

## 8 Detection and confirmation of amplicons

Amplicons from qualitative and quantitative reactions are detected by gel electrophoresis or through monitoring of the fluorescence signal by optical detection system (depending on the PCR format used) or any appropriate alternative.

During or after the PCR, the sequence of the amplicon should be verified to confirm the correct amplicon has been amplified. For example, the following methods can be used:

a) real-time PCR or dPCR using, for example, hybridization probes, hydrolysis probes or molecular beacons;

b) DNA sequencing of the amplicon;

c) restriction fragment length polymorphism (RFLP) analysis;

d) Southern blotting and probe hybridizations.

# 9 General environmental laboratory requirements

## 9.1 General

The facilities and environmental conditions shall be suitable for the laboratory activities and shall not adversely affect the validity of results according to the requirements for laboratory areas described in ISO 7218. Thus, the laboratory shall monitor, control and record environmental conditions in accordance with relevant specifications, methods or procedures or where they influence the validity of the results.

Critical environmental points in the PCR reaction are:

a) carry-over contamination, which is the contamination of samples by the dispersion of dust or aerosols carrying nucleic acid sequences from prior amplifications;

b) cross-contamination, which is the contamination of a sample with other samples due to the handling of highly concentrated nucleic acids (samples from the field or reference materials for example) in the same work areas of the samples to be analysed.

In order to minimize this risk of contamination, the different analytical steps where PCR activities are performed shall be organized taking into account the laboratory setup (unidirectional workflow, air flow, technical staff, equipment, samples, consumables, reagents, waste, laboratory clothing, etc.). These measures ensure that nucleic acid from the laboratory sample and amplified DNA generated by PCR remain physically separated.

Controls mentioned in Table 1 (see 12.2.2.1) shall be included to evaluate the risk of these contaminations.

## 9.2 Laboratory setup

### 9.2.1 General

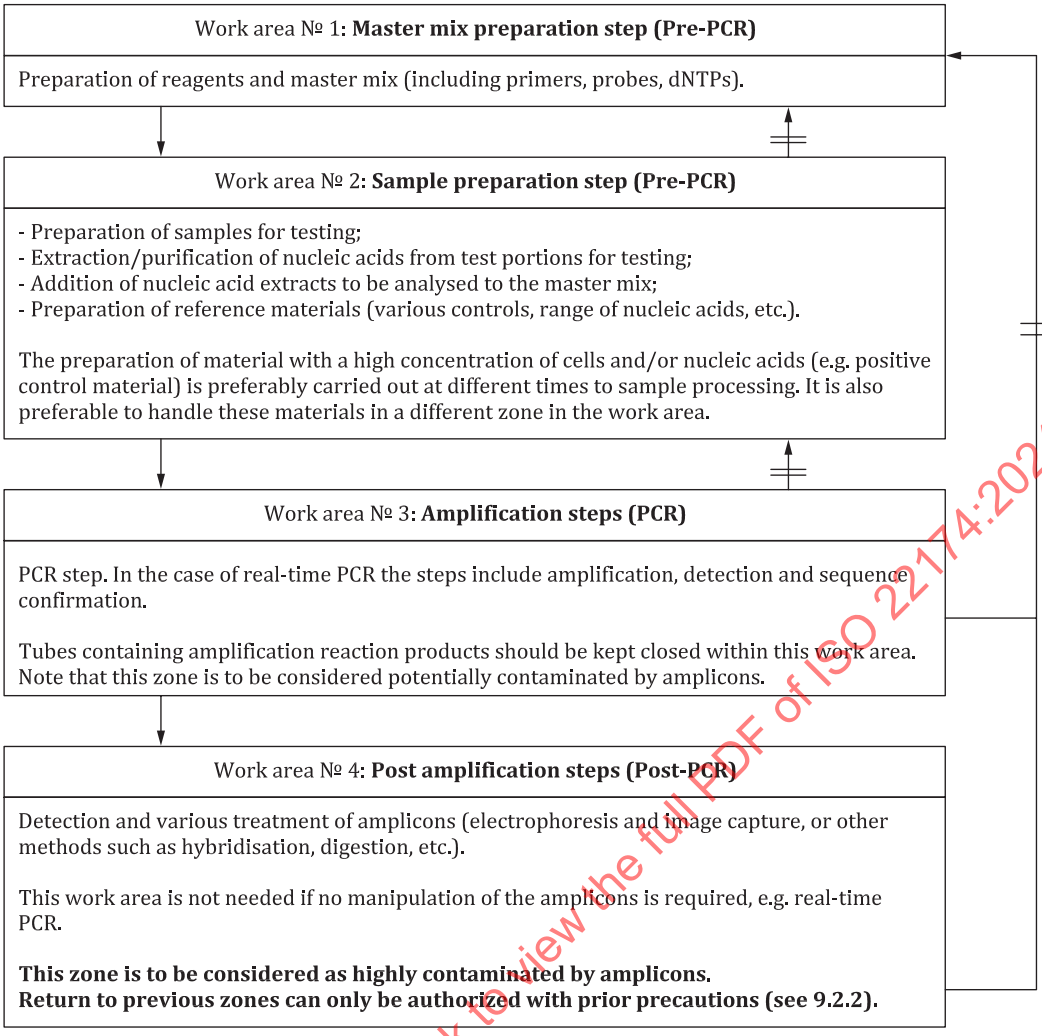
To prevent contamination of the reaction mixture by previously amplified target sequences, it shall be ensured that separate work areas (rooms and/or workstations) are available.

A work area is either:

a) a specific room, such as a closed room dedicated to one or more analytical phases; or

b) a workstation, such as a physically delimited zone on a workbench or ultraviolet (UV) cabinet, which can be part of a room, for the purposes of an analytical phase and having dedicated equipment and/or reagents specific to this phase.

A laboratory setup typically follows Figure 1, enabling physical separation by the use of three different zones. Physical separation through the use of different rooms is the most effective and preferable way of ensuring separate work areas and working facilities. However, considering the development of new tools or methodologies during the different steps of PCR analysis, e.g. partial or full automation process, the laboratory should decide on the need to adapt or simplify their implementation of physical separation zones.




**Key**

↓ authorized flow direction

⇊ authorized flow direction, but with prior precautions

Avoid negative air flow pressure in work area N° 1 and positive air flow pressure in work area N° 2, N° 3, N° 4.

**Figure 1 — Recommended organization flowchart for the use of facilities during PCR process**



### 9.2.2 Control of flows

#### 9.2.2.1 Air flows

The organization of air flows should not allow circulation of air from post-PCR areas to pre-PCR areas.

When possible, dynamic ambient air supply and removal circuits should be independent from one room to another.

#### 9.2.2.2 Personnel

A forward flow principle in space and/or time shall be adapted by the personnel. Dedicated sets of personal protective equipment should be used for the different work areas (e.g. laboratory coat, head covering, overshoes, gloves). Disposable gloves should be worn at sample preparation and when setting up PCR. Laboratory personal protective equipment shall be changed at appropriate frequencies. Movement of

personnel from post-PCR to pre-PCR work areas should only be possible after changing this personal protective equipment. Unfamiliar technical personnel shall be trained to the forward flow principle before working in these work areas.

#### 9.2.2.3 Circulation of documents, reagents, water, equipment, amplicons and waste

Cross-contamination events created by the transfer and storage of documents, reagents, consumables, equipment, samples, amplicons, waste, etc. shall be avoided. In particular, amplicons, positive controls and samples shall each be kept in a different chamber/container as those used for the storage of PCR reagents.

Single-use consumables and dedicated equipment (e.g. rack) in post-PCR work areas should be used. Tubes containing amplicons should not be cleaned or autoclaved or re-used after use to minimize the risk of contamination. Procedures for nucleic acid decontamination should be implemented before returning to a pre-PCR work area.

If the laboratory uses a water purification system, it should not be installed in work areas 3 and 4.

### 9.2.3 Cleaning of laboratory

Surfaces potentially contaminated by an infectious agent shall be disinfected. Surfaces potentially contaminated by nucleic acids shall be decontaminated using a nucleic acid denaturing solution (e.g. sodium hypochlorite). Detailed information about commonly used disinfectants, including the mode of action, microbial activity, effective concentrations and uses, is given in Reference [18].

Cleaning and maintenance personnel intervention procedures shall be described and adapted to the context and constraints of the laboratory. These personnel shall be informed about the restrictions and specific conditions of circulation.

### 9.2.4 Environmental monitoring for nucleic acid contamination

The laboratory work areas are monitored by the negative process, extraction and PCR controls, see Table 1. This kind of environmental control demonstrates the presence or absence of contamination during the performance of the test.

## 10 Reagents and consumables

Reagents and consumables used shall be of quality suitable for molecular biological applications (e.g. ultrapure water, reagents free from DNase and/or RNase), stored and used in appropriate conditions (storage, dilution, aliquoting, expiry, in accordance with supplier recommendations, etc.).

The laboratory should establish the list of reagents and consumables that are deemed critical in the full analytical process. It should describe the test procedures and monitor their conformity.

Testing may be performed either:

a) before their use in the analytical process; or

NOTE Generally, the new batches of reagents and/or consumables are tested by comparison with previous batches used. For example, internal reference controls (nucleic acid solution, biological samples, other reagents, etc.) can be used to test and validate the use of new batches of reagents.

b) by global post-auditing, i.e. using suitable controls to test the new batches used and their stability.

The acceptance of reagents and consumables used for the implementation of the full analytical process shall be documented.

## 11 Equipment

The laboratory shall use equipment that is suitable for the methods used.

In addition to standard laboratory equipment, the following equipment shall be used for PCR-based methods, with the exact selection of equipment being dependent on the format of PCR used (endpoint PCR, real-time PCR, dPCR).

**11.1 Thermal cycler,** which shall meet the temperature specifications required by the PCR-based methods used. Temperature calibration and performance testing of thermal cyclers shall be performed in accordance with ISO 20836.

NOTE Both accuracy and uniformity of the thermal cycler, during each of the temperature steps of the PCR protocol, determine the precision and accuracy of PCR results.

**11.2 System for detection of amplicons**, which comprises:

a) an apparatus for agarose or polyacrylamide gel electrophoresis and, if necessary, a UV radiation source for recording visualization of amplified DNA; or

b) an apparatus for nucleic acid column chromatography and the appropriate detection system; or

c) solid phases loaded with a specific probe and apparatus for detecting amplicons; or

d) other equally suitable systems.

**11.3 Real-time thermal cycler**, which shall meet the temperature and optical specifications as required by the real-time PCR-based methods used. Temperature calibration and performance testing of thermal cyclers shall be performed as described in ISO 20836.

At the time of publication of this document, there is no metrological traceable method available for optical calibration. The optical verification shall therefore be performed with a method of choice or as recommended by the manufacturer's instructions.

**11.4 Thermal cycler used for dPCR**, which shall meet the temperature and optical specifications as required by the dPCR methods used. Temperature calibration and performance testing of thermal cyclers shall be performed as described in ISO 20836 when the dPCR workflow includes a standard thermal cycler for amplification.

At the time of publication of this document, there is no metrological traceable method available for optical calibration. The optical verification shall therefore be performed with a method of choice or as recommended by the manufacturer's instructions.

**11.5 Pipettes (for all PCR formats).**

At least three sets of pipettes are required, one of each dedicated to:

a) sample preparation (pre-PCR zone, see Figure 1);

b) master mix preparation (pre-PCR zone, see Figure 1);

c) post-amplification steps (post-PCR zone, see Figure 1).

The calibration of pipettes is described in ISO 835 and ISO 8655-1.

## 12 Procedure

### 12.1 Enrichment and sample treatment

If enrichment of the test portion is required, it should be performed in accordance with the corresponding International Standards or other appropriate methods. Other enrichment media found to be more PCR compatible can be used, if they have been shown, through validation, to have performance at least comparable to those described in International Standards.

Some enrichment media contain fewer PCR-inhibitory substances than others, which should be carefully considered in connection with the choice of test portion preparation method.

For some products, special care should be taken to suppress the growth of competing background microorganisms (e.g. by addition of selective chemicals or antibiotics).

To avoid potential PCR inhibition, methods such as a simple dilution, centrifugation and immunoseparation can be applied. Physical methods can be used to reduce the fat content of high-fat samples.

## 12.2 Amplification

### 12.2.1 General

Add the nucleic acid solution to the appropriate reaction mixture according to the method and carry out the remaining steps of the PCR using appropriate temperature/time profile and cycle number for the detection system (primer for endpoint PCR or primer with probe for real-time format).

### 12.2.2 Control reaction

#### 12.2.2.1 General

The appropriate positive, negative and internal/external controls for each step shall be selected according to a defined rationale. A description of the controls for detection and quantification of microorganisms by PCR is given in Table 1.

**Table 1 — Controls for PCR analysis**

| Step | Negative process control[a] | Positive process control[a] | Internal process control[b] | Negative extraction control[c] | Internal/external amplification control[d] | Positive PCR control[e] | Negative PCR control[e] |
|---|---|---|---|---|---|---|---|
| Sample treatment | ↓ | ↓ | ↓ | | | | |
| Nucleic acid extraction | ↓ | ↓ | ↓ | ↓ | | | |
| Amplification | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| Detection | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |

**Key**

↓ Procedures covered by this control

[a] The frequency of use shall be determined as part of the laboratory quality assurance programme.

[b] For each quantitative analysis, the internal process control shall be used. For qualitative analyses, it is recommended to use this control at regular intervals determined by the laboratory.

[c] This control shall be used at all times except when the negative process control is performed.

[d] The internal or external amplification control shall be performed for every nucleic acid extract except if an internal process control is used.

[e] This control is necessary for every sample series in a PCR run.

#### 12.2.2.2 Negative process control

The negative process control is used to determine if contamination of the samples could have occurred during any stage of the process. For this control, a reagent/component that is free of target nucleic acid shall be used (e.g. ultrapure water, BPW or peptone salts). It is also possible to use the same matrix as the analysed test samples for this control; however, in this case, the matrix shall be shown to be target nucleic acid sequence free. This control shall give a negative result.

The frequency of use shall be determined as part of the laboratory quality assurance programme.

#### 12.2.2.3 Positive process control

The positive process control is used to determine if the complete process from sample treatment until nucleic acid amplification has been carried out without any problems. For this control, a sample spiked with the target microorganism or its nucleic acid is processed in the same way as the test samples. Care should be taken to avoid false positive results through contamination by this control.

The frequency of use shall be determined as part of the laboratory quality assurance programme.

#### 12.2.2.4 Internal process control

Internal process controls can be an endogenous sequence (from a genomic sequence naturally present in the tested matrix) or an exogenous sequence (from a genomic sequence naturally absent in the tested matrix).

The internal process control can be used to cover both the extraction and amplification steps. It is used to determine the efficiency of the nucleic acid extraction and to measure by how much the PCR reaction is inhibited. The internal process control consists of a known amount of material (DNA, RNA, bacterial or viral strain) which is naturally absent in the tested matrix. The internal process control is added directly to each sample including the negative process control and positive process control to undergo the complete procedure.

For each quantitative analysis, the internal process control shall be used. For qualitative analyses, it is recommended to use this control at regular intervals determined by the laboratory. How often this is needed shall be determined on a case-by-case basis.

When using a homologous internal process control, optimization of the concentration should be performed prior to commencing analysis, to ensure that any competition between the internal process control and the microbial target sequence is minimized.

#### 12.2.2.5 Negative extraction control

The negative extraction control is used to determine if it is possible that contamination of the samples has occurred during the extraction and amplification stages of the process. For this control, a reagent/component that is free of target nucleic acid shall be used (e.g. ultrapure water, BPW or peptone salts). It is also possible to use the same matrix as the analysed test samples for this control; however, in this case, the matrix shall be shown to be target nucleic acid sequence free. This control shall give a negative result.

This control shall be used at all times except when the negative process control is performed.

#### 12.2.2.6 Amplification control

The amplification control is used to determine if, and by how much, the PCR is inhibited.

An internal amplification control is added directly to the master mix of each sample including the negative PCR control, the negative process control and the positive process control. When no target signal is observed, the internal amplification control shall give the expected positive result previously defined by the laboratory.

Where an external amplification control is used, the sample nucleic acid extract is split into separate aliquots. The external amplification control is added to one aliquot, and each aliquot is run in parallel. The external amplification control shall give a positive result.

An amplification control shall always be used during PCR-based methods when no internal process control is used. The internal process control can also function as an amplification control.

When using a homologous internal amplification control, optimization of the concentration should be performed prior to commencing analysis, to ensure that any competition between the internal amplification control and the microbial target sequence is minimized.

NOTE The result of the external amplification control cannot definitively determine the amplification process has worked correctly in the test reaction.

#### 12.2.2.7 Positive PCR control

The positive PCR control is used to determine if the PCR reaction has been performed without any problems. For the positive PCR control, the target nucleic acid is added to the reaction. This control shall give a positive result. Care should be taken to avoid false positive results through contamination by this control.

This control shall be used in every PCR that is run.

NOTE A positive control can consist of a plasmid containing the target sequence(s) or nucleic acid sequence artificially constructed or derived from the target microorganism.

#### 12.2.2.8 Negative PCR control

The negative PCR control is used to determine if contamination of the PCR reagents has occurred. Ultrapure water (or other PCR-inert substrate such as grinding or elution buffer) is added to the reaction instead of the nucleic acid template. This control shall give a negative result.

This control is necessary for every sample series in a PCR run.

### 12.2.3 Detection of amplicon

Amplicons can be detected after the amplification step using a technique of choice (e.g. gel electrophoresis, chromatography).

In real-time PCR amplicons are detected during or after the amplification via the optical unit of the real-time thermal cycler. After the amplification step, the data are analysed (see 12.2.4).

### 12.2.4 Data analysis

#### 12.2.4.1 Real-time PCR

Real-time PCR data analysis is performed by the real-time thermal cycler software. The aim is to determine the quantification cycle ($C_q$) for each individual real-time PCR. There are several different methods of $C_q$ determination, depending on the software and hardware used to acquire and analyse real-time PCR data. Whichever method is used, the user should ensure that the $C_q$ obtained is within the exponential phase of the reaction and above baseline noise. For methods using a baseline setting, the baseline should be set to the region of the amplification curve prior to exponential amplification and without abrupt or large changes in fluorescence (e.g. cycles 3 to 15). See Annex A for more information on amplification curves.

These recommendations apply to both automatic and manual threshold setting. Presence of the target shall be confirmed by comparing the obtained $C_q$ of the test portion to the previously established $C_q$ for the LOD of the test. Care shall be taken when comparing $C_q$ between instruments (even between the same brand and model) as each individual instrument can produce a different $C_q$ for the same target concentration.

#### 12.2.4.2 dPCR

The software associated to the reader or the full-integrated apparatus performs data analysis. After amplification, the fluorescent signal is read (end-point PCR) and each partition is noted as negative or positive (digital reading). A Poisson-law-based statistical analysis should be used for direct estimation of the target nucleic acid quantification.

This nucleic acid quantification based on statistical adjustment shall follow the following set of assumptions:

— target nucleic acids are randomly distributed over the total number of partitions analysed;

— presence of the target leads to a positive classification of the partition;

— absence of the target leads to a negative classification of the partition;

— the number of copies of the target sequence in the genome shall be known;

— all partitions have the same volume.

For each dPCR reaction:

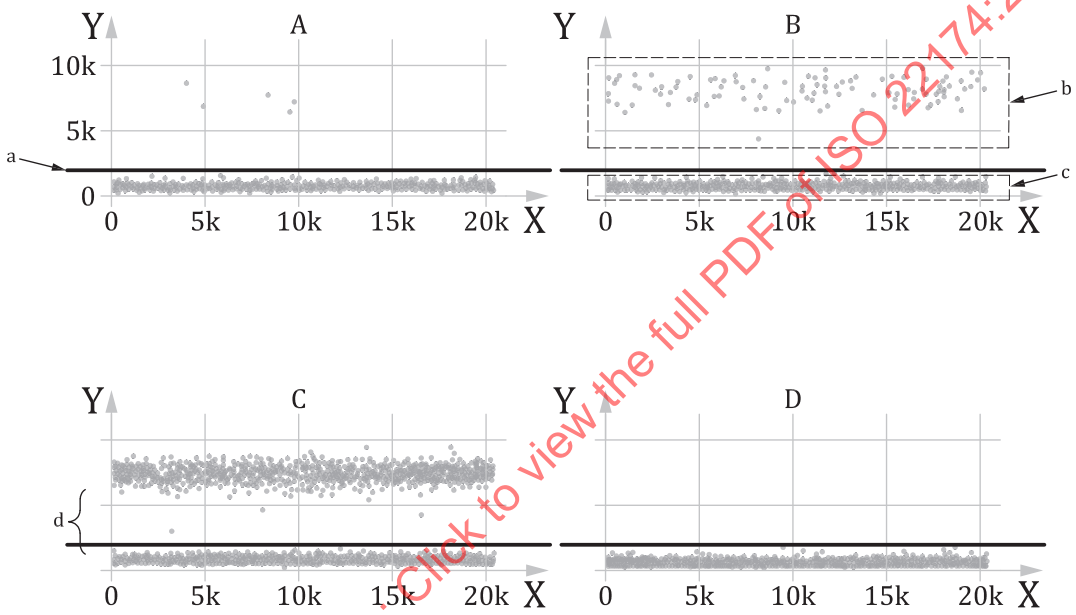
— check the passive reference and/or that the number of valid partitions conforms to the supplier's recommendations;

— check the separability between negative and positive clusters for each sample;

— check that negative controls are within the limit of blank;

— check that the quantification of the positive control(s) conforms to the expected value/range.

The threshold limit should be set above the highest point of the negative cluster from the negative control(s): the threshold position can be set automatically or manually (see Figure 2).




**Key**

X partition
Y fluorescence intensity
A sample 1
B sample 2
C sample 3
D negative PCR control

a Analysis threshold.
b Positive cluster.
c Negative cluster.
d Separability.

**Figure 2 — 1D scatter plots illustration of a serial dilution of a positive sample and its negative PCR control**



## 12.3 Evaluation

### 12.3.1 Qualitative evaluation

Evaluation is possible provided the results obtained with the controls specified in this subclause are unambiguous.

Examples of PCR results are given in Table 2. Other results among controls can occur; these shall be evaluated on a case-by-case basis following a root cause analysis.

**Table 2 — Examples of possible PCR results**

| Target signal | Positive process control | Positive PCR control | Negative process control/ Negative extraction control/ Negative PCR control | Internal process control | Internal/ external amplification control | Interpretation of results |
|---|---|---|---|---|---|---|
| + | + | + | – | + | +/– | Target amplicon detected |
| – | + | + | – | + | + | No target amplicon detected |
| + | + | + | + | +/– | +/– | Inconclusive[a] |
| – | – | + | – | – | – | Inconclusive[b] |
| +/– | +/– | – | – | +/– | +/– | Inconclusive[c] |

[a] Possible contamination.

[b] Possible inhibition.

[c] Possible error during master mix or PCR setup.

### 12.3.2 Quantitative evaluation

#### 12.3.2.1 General

For qPCR, further analysis of the $C_q$ values shall be performed after evaluation of the PCR result validity (see 12.3.1).

A standard curve shall be constructed by a minimum of four different concentrations from the standard material, each in at least duplicate. Do not average $C_q$ values from duplicate reactions prior to plotting. In these cases, check $C_q$ values of the standard curve for any outlying values and remove these from the series. No more than two such outlying $C_q$ values shall be removed per series and values from a minimum of four concentrations shall be retained.

The logarithm of the concentration of the standards is plotted along the x-axis and their $C_q$ values plotted along the y-axis. After a linear regression analysis, the quantity of an unknown sample can be derived from the resulting equation and obtained $C_q$ value. The dynamic range of the standard curve shall cover the values expected for the specific application. The amplification efficiency shall be between 90 % to 110 % (−3,6 ≥ slope ≥ −3,1). The $R^2$ coefficient shall be ≥ 0,98.

#### 12.3.2.2 Absolute quantification

The concentration of target sequences in the samples is determined for positive samples using an approach dependent on the PCR-based method used, as follows:

a) Absolute quantification by real-time PCR: The concentration is calculated by comparing the $C_q$ value of the unknown reaction to the $C_q$ values of a standard curve prepared from standard materials of known concentration. This calculation may be carried out directly by the real-time PCR thermal cycler software, or by the analyst using, for example, calculation spreadsheets or qPCR dedicated software application designed and validated for the purpose.

b) Absolute quantification by dPCR: The dPCR thermal cycler software automatically calculates this concentration by applying corrective statistics to the number of positive and negative partitions for each reaction. If the target sequence concentration is given for the reaction mix by the software, a back calculation is required to obtain the concentration in the nucleic acid extract.

For both absolute quantification approaches, the concentration of target sequences in the nucleic acid extract is converted into a final quantitative result for the sample in units of, for example, cfu/copies/genome

equivalents per g/ml/cm$^2$ (dependent on the requirements of the target organism/matrix combination) by application of appropriate concentration and other conversion factors.

#### 12.3.2.3 Relative quantification

Results are calculated as the ratio of abundance of the target microorganism in the sample under test relative to its abundance in a reference material, as follows:

a) Relative quantification by real-time PCR: The calculation can be carried out using the following standard curve or comparative $C_q$ methods:

   1) In the standard curve method, the $C_q$ values for the target sequence in both the sample under test and the reference material are compared to the $C_q$ values of a standard curve prepared from material containing the target sequence.

   2) In the comparative $C_q$ method, the calculation takes into account the $C_q$ values for both the target sequence and a reference sequence in both the sample under test and the reference material.

b) Relative quantification by dPCR: Concentrations of target microorganism are directly obtained by applying corrective statistics to the number of positive and negative partitions found in the samples (tested and reference material). Comparison of these results allow the determination of the ratio.

NOTE ISO/IEC 17025 recommends the usage of certified reference material manufactured by producers fulfilling the requirements of ISO 17034.

### 12.4 Test report

The test report of the specific standard shall be followed.

## 13 Performance characteristics of PCR-based methods

Performance characteristics of PCR-based methods are specified in ISO 22118.

For methods that include a PCR-based detection step, the relevant performance characteristics of the PCR step should first be assessed based on ISO 22118, before entering the phase of validation of the complete analytical procedure following the ISO 16140 series.

## 14 Validation and verification of PCR-based methods

### 14.1 General

Both validation and verification of microbiological methods are discussed in detail in the ISO 16140 series and ISO 17468.

Performance characteristics of standardized reference methods are determined by validation studies and have been included in some International Standards. These enable a user laboratory to verify their own performance by comparing the published characteristics with their own results.

### 14.2 Validation

Validation of PCR-based methods aims to ensure that routine testing will meet the established method performance characteristics and therefore will be close to the unknown “true” value.

By definition, validation is the “establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled” (see ISO 16140-1:2016, 2.81).

Laboratories may use reference methods, standardized reference methods, alternative (proprietary) methods or in-house developed methods. If a method has been validated and relevant performance