



BSI Standards Publication

**Soil quality — Estimation of abundance
of selected microbial gene sequences by
quantitative polymerase chain reaction (qPCR)
from DNA directly extracted from soil**

National foreword

This British Standard is the UK implementation of EN ISO 17601:2025. It is identical to ISO 17601:2025. It supersedes BS EN ISO 17601:2018, which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/4, Soil quality.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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English Version

Soil quality - Estimation of abundance of selected
microbial gene sequences by quantitative polymerase
chain reaction (qPCR) from DNA directly extracted from
soil (ISO 17601:2025)

Qualité du sol - Estimation de l'abondance de
séquences de gènes microbiens par amplification par
réaction de polymérisation en chaîne quantitative
(qPCR) à partir d'ADN directement extrait du sol (ISO
17601:2025)

Bodenbeschaffenheit - Ermittlung der Häufigkeit
ausgewählter mikrobieller Gensequenzen durch
quantitative PCR aus DNA-Boden-Extrakten (ISO
17601:2025)

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

European foreword

This document (EN ISO 17601:2025) has been prepared by Technical Committee ISO/TC 190 "Soil quality" in collaboration with Technical Committee CEN/TC 444 "Environmental characterization of solid matrices" the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2026, and conflicting national standards shall be withdrawn at the latest by May 2026.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN ISO 17601:2018.

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Endorsement notice

The text of ISO 17601:2025 has been approved by CEN as EN ISO 17601:2025 without any modification.

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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).

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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 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 17601:2016), which has been technically revised.

The main changes are as follows:

- [Annex C](#) has been expanded by adding examples of well-established qPCR systems to quantify certain microbial groups or their function.

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

DNA (DNAs) is a major component of any living organism, coding for enzymes responsible for their biological activities. The study of DNA sequences from DNA sources extracted from different environmental matrices, by means of numerous molecular approaches, provides molecular markers that can be used to sharply distinguish and identify different organisms (bacteria, archaea, and eukaryotes).

Up to now, most of the studies aiming to develop microbial quality indicators applicable to complex environment such as soil were biased by the poor culturability of many microorganisms under laboratory conditions and the lack of sensitivity of traditional microbiological methods. The recent development of a large set of molecular biology methods based on amplification of soil-extracted nucleic acids have provided a pertinent alternative to classical culture-based microbiological methods providing unique insight into the composition, richness, and structure of microbial communities.^{[2][3][4][5][6]} DNA-based approaches are now well established in soil ecology and serve as genotypic markers for determining microbial diversity. The results of molecular analyses of soil microbial communities and populations rely on two main parameters: a) the extraction of DNA representative of the indigenous bacterial community composition, and b) PCR bias such as the choice of primers, the concentration of amplified DNA, errors in the PCR, or even the method chosen for the analysis.^{[7][4][8][9]}

Numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils.^[10] Recently, ISO 11063 reported “a method to extract nucleic acids directly from soil samples” derived from Reference ^[10], opening a new window for developing standardized molecular approaches to estimate soil quality.^[11]

The aim of this document is to describe the procedure used to set up and perform quantitative PCR to quantify the abundance of soil microbial phyla, as well as functional groups, from DNA directly extracted from soil samples. The quantification of soil microbial phyla and functional groups by qPCR assays can contribute to the development of routine tools to monitor soil quality. The repeatability and the reproducibility of the qPCR procedure were assessed in an international ring test study (see [Annex B](#)). The repeatability of this procedure was successfully evaluated for 16S rRNA genes and for genes coding a functional marker of denitrifiers (the nitrite reductase gene *nirK*). The reproducibility of this procedure revealed a laboratory effect which can be overcome by interpreting the results of the quantification of the abundance of the microbial groups by comparison, either by using an external reference (DNA extracted from a control strain) in the assay or by calculating a percentage of variations between treatments to normalize the data. It is noteworthy that the number of genes is not necessarily directly linked to the number of organisms that are measured. For example, the number of ribosomal operon is ranging from one copy to 20 copies in different bacterial phyla. Therefore, the number of 16S rRNA sequences quantified from soil DNA extracts does not give an exact estimate of the number of soil bacteria. Furthermore, the number of sequences is not necessarily linked to living microorganisms and can comprise sequences amplified from dead microorganisms.

A list of currently well-established qPCR assays to assess selected functional traits of the soil microbiome is listed in [Annex C](#).

Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative polymerase chain reaction (qPCR) from DNA directly extracted from soil

1 Scope

This document specifies the crucial steps of a quantitative polymerase chain reaction (qPCR) method to measure the abundance of selected microbial gene sequences from soil DNA extract. The number of microbial gene sequences quantified by qPCR provides an estimation of the abundance of selected microbial groups in soil.

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 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 11063, *Soil quality — Direct extraction of soil DNA*

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 soil DNA

DNA extracted from living and dead biota in the soil

Note 1 to entry: The soil biota includes microorganisms, plants and animals.

3.2 polymerase chain reaction PCR

method allowing the amplification of a specific DNA sequence using a specific pair of oligonucleotide primers

3.3 quantitative polymerase chain reaction qPCR

method allowing the quantification in a DNA *template* (3.4) of the number of DNA sequences targeted by a specific pair of oligonucleotide primers

3.4 template

DNA sample used to perform *polymerase chain reaction* (PCR) (3.2) to amplify a specific DNA sequence

3.5

amplicon

PCR product obtained by *polymerase chain reaction (PCR)* (3.2) from a *template* (3.4)

3.6

cloning vector

circular DNA molecule in which the *amplicon* (3.5) is inserted by ligation and which is used to transform competent *Escherichia coli*

3.7

qPCR standard

cloned DNA target used as *template* (3.4) for *quantitative polymerase reaction (qPCR)* (3.3) to establish the standard curve relating the abundance of target sequence as a function of *cycle threshold (Ct)* (3.9) values

3.8

non-template control

NTC

control, usually molecular grade water, that is used as negative control in *quantitative polymerase reaction (qPCR)* (3.3) assay to check for the absence of contaminant in the qPCR mix

3.9

cycle threshold

Ct

number of *quantitative polymerase chain reaction (qPCR)* (3.3) cycles required for the fluorescent signal to cross the threshold (i.e. to exceed background level)

Note 1 to entry: The Ct value is inversely proportional to the abundance of the target sequence.

4 Principle

This document describes a qPCR assay using fluorescent DNA binding dye as reporter. This qPCR assay has been validated by an international ring test conducted with the SYBR Green¹⁾, a commonly used fluorescent DNA binding dye which binds all double-stranded DNA and can be detected by measuring the increase in fluorescence throughout the cycle.

The method comprises four tasks and eight steps as summarized in [Figure 1](#). According to Reference [1], the three critical steps to be validated for each qPCR assay are as shown in [Figure 1](#).

1) SYBR® Green is a trademark of Thermo Fisher Scientific Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

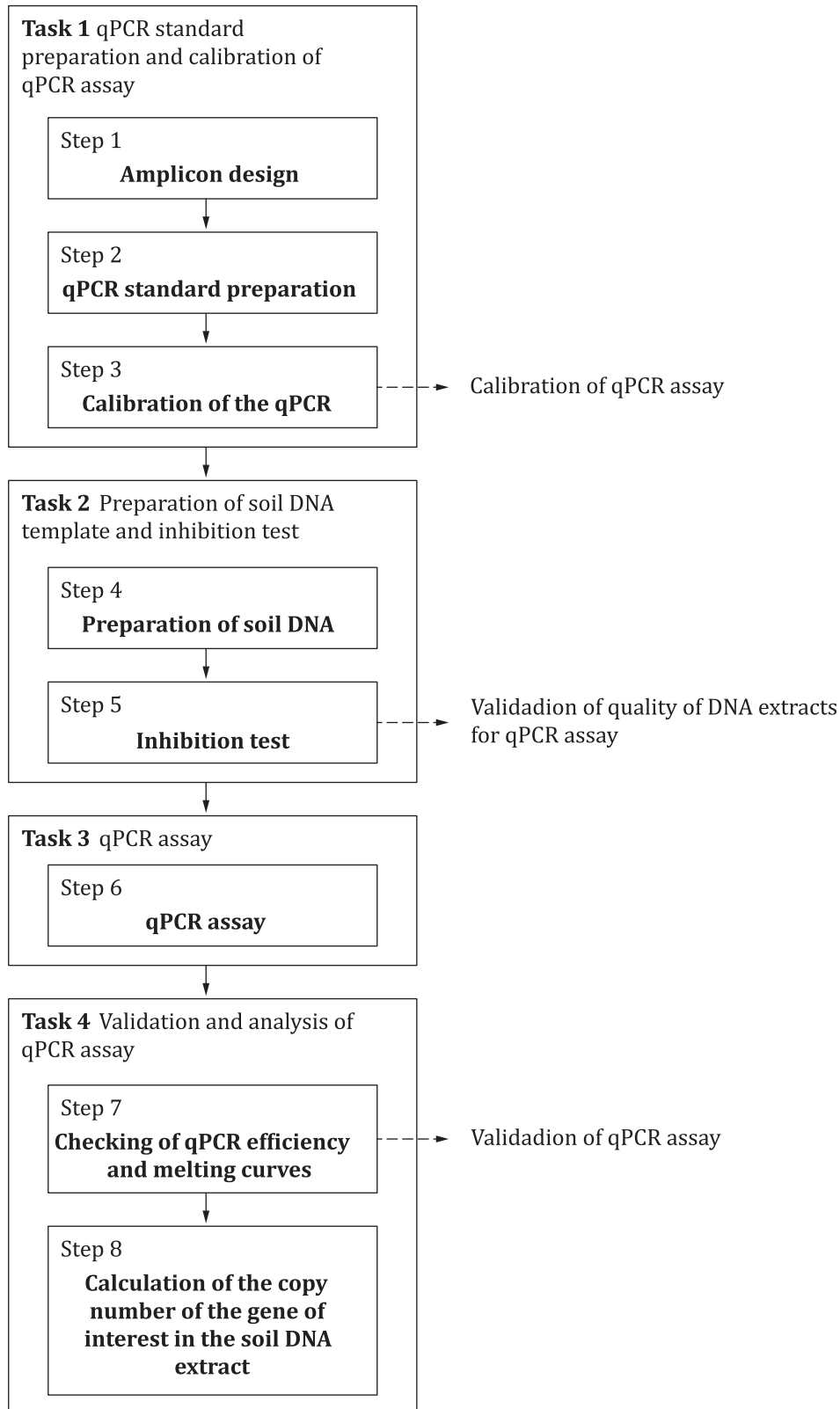


Figure 1 — Main tasks and critical steps to estimate the abundance of selected microbial gene sequences by qPCR assay

This document describes a qPCR assay based on the use of fluorescent DNA binding dye which has been validated by an international ring test using SYBR Green qPCR. In [Annex A](#), information about the TaqMan®²⁾ qPCR assay (which was not tested in the international ring test) is given. The first task comprises three steps describing the design of optimal amplicon for qPCR (step 1), the preparation of qPCR standards (step 2), and the procedure to calibrate the qPCR assay (step 3). The second task includes two steps describing the procedures to prepare soil DNA samples (step 4) and to test for the presence of qPCR inhibitors in soil DNA samples (step 5). The third task is constituted of a single step describing the protocol to perform the qPCR assay (step 6).

Finally, the fourth task is made of two steps: one describes the procedure to validate the qPCR assays (step 7) to check its quality and the other describes the different options to calculate the copy number of gene sequences from the cycle threshold values (Ct) obtained from the analysis of qPCR amplification plots (step 8).

5 Test materials

5.1 DNA

5.1.1 DNA, extracted from pure bacterial and fungal isolates using classical extraction procedures or by using commercial kit to extract genomic DNA.

5.1.2 Soil DNA, extracted from aliquots of soil according to ISO 11063.

5.2 Bacteria

5.2.1 *Escherichia coli* strain, usually used for cloning of PCR product.

5.3 Plasmid

5.3.1 Cloning vector, usually used for cloning of PCR product in competent *Escherichia coli*.

5.4 Enzymes

5.4.1 Taq polymerase.

5.4.2 T4 DNA ligase.

5.4.3 T4 gene 32 protein (T4gp32).

5.4.4 Bovine serum albumin (CAS RN³⁾ 9048-46-8).

5.5 Chemicals

5.5.1 Ampicilline sodium, C₁₆H₁₈N₃NaO₄S (CAS No. 69-52-3).

5.5.2 Boric acid, BH₃O₃ (CAS No. 10043-35-3).

2) TaqMan is a trademark of Roche Molecular Systems, Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3) Chemical Abstract Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.5.3 Deoxynucleotide solution, dNTPs.

5.5.4 SYBR Safe®⁵⁾ double-strand DNA gel stain.

5.5.5 Ethylenediaminetetraacetic acid disodium salt (EDTA) dihydrate, $C_{10}H_{14}N_2O_8Na_2 \cdot 2 H_2O$ (CAS No. 6381-92-6).

5.5.6 Glucose, $C_6H_{12}O_6$ (CAS No. 50-99-7).

5.5.7 Hydrochloric acid, HCl (CAS No. 7647-01-0).

5.5.8 IPTG, Isopropyl-Beta-D-Thiogalactopyranoside, (CAS No. 367-93-1).

5.5.9 Magnesium chloride, $MgCl_2$ (CAS No. 7786-30-3).

5.5.10 Magnesium sulfate, $MgSO_4$ (CAS No. 7487-88-9).

5.5.11 Molecular-biology-grade water, H_2O .

5.5.12 Potassium chloride, KCl (CAS No. 7447-40-7).

5.5.13 Sodium chloride, NaCl (CAS No. 7647-14-5).

5.5.14 Tris[hydroxymethyl]aminomethane, $C_4H_{11}NO_3$ (CAS No. 77-86-1).

5.5.15 X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, (CAS No. 7240-90-6).

5.6 Products for bacterial culture medium

5.6.1 Bacto™tryptone⁶⁾, enzymatic digest of casein.

5.6.2 Yeast extract powder (CAS No. 8013-01-2).

5.7 Buffers and reagents

5.7.1 Ampicillin solution, 2 g of ampicilline sodium in 4 ml of 0,22 µm filter sterilized H_2O . Adjust to 20 ml with sterilized H_2O , prepare 1 ml aliquots, and store at $-20\text{ }^{\circ}\text{C}$.

5.7.2 EDTA, $0,5\text{ mol}\cdot\text{l}^{-1}$, 186,10 g of EDTA in 1 000 ml of H_2O adjusting with NaOH ($10\text{ mol}\cdot\text{l}^{-1}$) to pH 8,0.

5.7.3 SYBR Safe® DNA gel stain, dilute $10\ 000 \times$ SYBR Safe® gel stain in TBE buffer $\times 1$.

5.7.4 IPTG stock solution, 1 g of IPTG in 8 ml of H_2O . After careful mixing, the solution is adjusted to 10 ml and sterilized under security microbiology post. Prepare 1 ml aliquot of IPTG and store at $-20\text{ }^{\circ}\text{C}$.

5.7.5 Solid LB medium, 10 g of Bacto tryptone®, 5 g of yeast extract, 5 g of sodium chloride, and 15 g of agar in 1 000 ml of H_2O . After autoclaving for 20 min at $120\text{ }^{\circ}\text{C}$, 1 ml of ampicillin stock solution at $100\text{ mg}\cdot\text{ml}^{-1}$ is added to LB medium and plated in Petri dishes (20 ml) under a security microbiology post. 100 µl of IPTG solution are plated on solid LB-ampicillin medium. When IPTG solution is entered in LB-ampicillin medium, 20 µl of X-Gal solution is plated on solid LB-ampicillin medium. Solid LB medium is stored at $4\text{ }^{\circ}\text{C}$ until its use.

5.7.6 SOC medium, 20 g of Bacto tryptone®, 5 g of yeast extract, 0,58 g of NaCl, 0,95 g of MgCl₂, 2,46 g of MgSO₄, and 3,60 g of glucose in 1 l H₂O. Sterilize by 20 min autoclaving at 120 °C. Prepare 950 ml aliquots and store at -20 °C.

5.7.7 Tris-HCl, 1 mol·l⁻¹, 121,14 g of Tris in 1 000 ml of H₂O adjusting with 4 mol·l⁻¹HCl to pH 8,0.

5.7.8 TBE buffer × 10, pH 8,0, 108 g of Tris base, 55 g of boric acid, and 40 ml of 0,5 mol·l⁻¹EDTA (pH 8,0) in 1 000 ml of H₂O.

5.7.9 TBE buffer × 1, 100 ml of TBE buffer × 10 in 900 ml of H₂O.

5.7.10 TE buffer × 10, pH 8,0, 100 ml of 1 mol·l⁻¹Tris-HCl pH 8,0, 20 ml of 50 mmol·l⁻¹ EDTA pH 8,0 in 880 ml of molecular grade water.

5.7.11 TE buffer × 1, 100 ml of TE buffer × 10 in 900 ml of H₂O.

5.7.12 X-gal solution, 250 mg of X-Gal in 5 ml of dimethylformamide. After careful mixing, prepare 0,5 ml aliquot and store at -20 °C.

6 Apparatus

Use standard laboratory equipment including pipettes, a centrifuge, fume hood cabinet, horizontal electrophoresis system and the following.

6.1 Thermocycler suitable for qPCR analysis, allowing the real-time quantification of amplicons from various DNA templates with a theoretical detection limit of one copy of a sequence target per sample analysed.

6.2 Spectro-photometer, allowing the quantification of double-strand DNA at 260 nm.

6.3 Spectro-fluorimeter, allowing the quantification of double-strand DNA.

NOTE Only one of these two apparatus is required to estimate DNA concentration.

7 Procedure

7.1 qPCR standard preparation and calibration of qPCR assay (task 1)

7.1.1 General

The qPCR assay is based on the quantification of the amplicons at the end of each PCR cycle by using a DNA dye which fluoresces when intercalated in the double strand amplicons. The purpose of this task is to describe the definition of the appropriate amplicon to settle down a qPCR assay (step 1), the preparation of the qPCR standard (step 2), and the calibration of the qPCR assay (step 3).

7.1.2 Amplicon design (task 1, step 1)

7.1.2.1 General

The first step aims at designing the oligonucleotide primer pair; it can be designed in silico using different programs using the sequence of the microbial gene of interest to be quantified by qPCR from soil DNA extracts. The specificity of the primers shall be checked in silico by comparing their sequences to known sequences