DRAFT INTERNATIONAL STANDARD

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Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative realtime PCR from DNA directly extracted from soil

Qualité du sol — Estimation de l'abondance de séquences spécifiques de gènes microbiens par amplification en chaîne par polymerase (PCR) quantitative en temps réel à partir d'ADN directement extrait du sol

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 17601 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

This second/third/... edition cancels and replaces the first/second/... edition (), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / have] been technically revised.

Introduction

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

Up to now, most of the studies aiming to develop microbial soil quality indicators applicable to complex environment, such as soil, were biased by the unculturability of many microorganisms and the lack of sensitivity of traditional microbiological methods [1]. The recent development of numerous molecular biology methods based primarily 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 (= molecular genetic) markers for determining microbial diversity. The results of molecular analyses of soil microbial communities and/or populations rely on two main parameters: (i) the extraction of DNA representative of the indigenous bacterial community composition and (ii) PCR bias, such as the choice of primers, the concentration of amplified DNA, errors in the PCR, or even the method chosen for 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 reporting "a method to extract nucleic acids directly from soil samples„ derived from [10] is opening a new window for developing standardized molecular approaches to estimate soil quality [11].

The aim of this International Standard 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 as well as functional groups by qPCR assays can contribute to the development of routine tools to monitor soil quality. At the demand of ISO, the reproducibility of the quantification of the abundance of selected microbial gene sequences by quantitative realtime PCR from DNA directly extracted from soil was assessed in an International ring test study (Annex A).

Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative realtime PCR from DNA directly extracted from soil

1 Scope

2 Scope

This International Standard specifies the crucial steps of a quantitative real-time polymerase chain reaction (qPCR) method to measure the abundance of selected microbial gene sequences from soil DNA extract which provides an estimation of selected microbial groups.

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.

3 Normative references

The following referenced documents are indispensable for the application 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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the 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 — Method to directly extract DNA from soil samples*

4 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
soil DNA
DNA extracted from soil of living and dead biota

EXAMPLE Microorganisms plants, 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 realtime polymerase chain reaction
qPCR
method allowing the quantification in a template of the number of a specific DNA sequence using a specific pair of oligonucleotide primers

3.4.
template
DNA sample used to perform PCR to amplify a specific DNA sequence

3.5
amplicon
PCR product obtained by PCR from a template

3.6
cloning vector
circular DNA molecule in which the amplicon is inserted by ligation used to transform competent *Escherichia coli* for cloning the amplicon

5 Principle

The method aims to measure the abundance of selected microbial gene sequences from soil DNA extract. The method comprised four tasks and eight steps as shown in Figure 1.

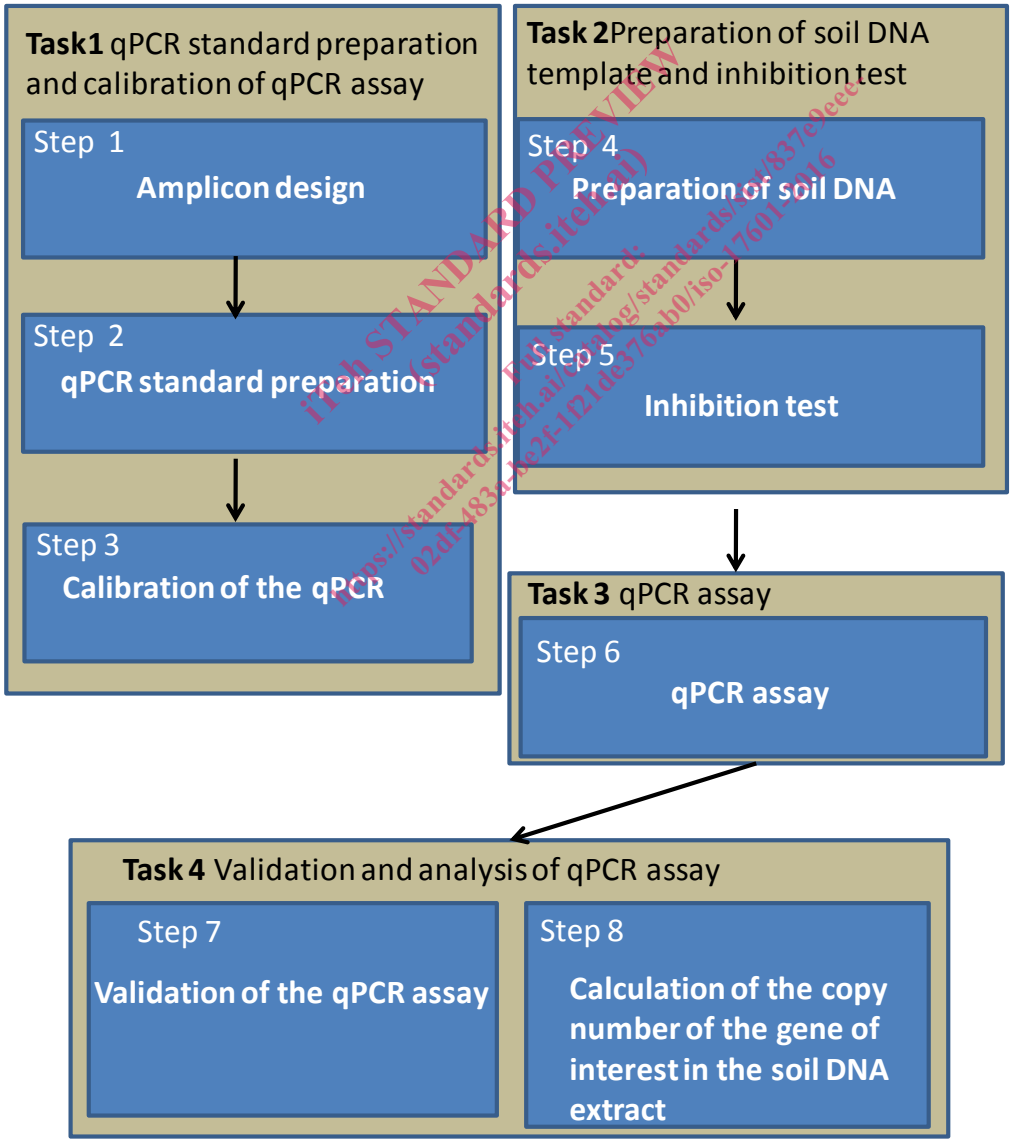

Figure 1 — Main tasks to estimate the abundance of selected microbial gene sequences by qPCR

The first task is made of three steps describing the design of optimal amplicon for qPCR (step one), the preparation of qPCR standards (step two) and the procedure to calibrate the qPCR assay (step three). The second task includes two additional steps describing the procedures to prepare soil DNA samples (step four) and to test for the presence of qPCR inhibitors in soil DNA samples (step five). The third task is constituted of a single step describing the protocol to perform SYBR®[1)] Green and TaqMan®[2)] qPCR assays (step six). Finally, the fourth task is made of two steps, one describing the procedure to validate qPCR assays (step 7) to check for the quality of qPCR assay and another one describing the different options to calculate the number of sequences of the gene of interest copy from cycle threshold (Ct) obtained from the analysis of qPCR amplification plots (step 8).

6 Test materials

6.1 DNA

5.1.1 **DNA** is 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** is extracted from aliquots of soil according to ISO 11063.

6.2 Bacteria

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

6.3 Plasmid

5.3.1 **Cloning vector,** usually used for cloning of PCR product in competent *Escherichia coli,* having SP6 and T7 universal primers on both site of the polylinker.

6.4 Enzymes and proteins

5.4.1 Taq polymerase.

5.4.1 T4 DNA ligase.

5.4.3 T4 gene T32 protein.

5.4.4 Bovine serum albumin (CAS No. 9048-46-8).

6.5 Chemicals

5.5.1 **Amipicilline sodium**, $C_{16}H_{18}N_3NaO_4S$ (CAS No. 69-52-3).

5.5.2 **Boric acid**, BH_3O_3 (CAS No. 10043-35-3).

5.5.3 **Deoxynucleotide solution**, dNTPs.

5.5.4 **Ethidium bromide** (CAS No. 1239-45-8).

1) SYBR Green is a registered trademark of Molecular Probes. 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.

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.

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

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

5.5.7 Chlorhydric 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).

6.6 Product for bacterial culture medium

5.6.1 Bacto®3) tryptone, enzymatic digest of casein.

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

5.6.3 Agar

6.7 Buffer and reagents

5.7.1 Ampicilline 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 °C.

5.7.2 EDTA, 0,5 mol/l, 186,10 g of EDTA in 1 000 ml of H_2O, adjusting with NaOH (10 mol/l) to pH 8,0.

5.7.3 Ethidium bromide, 5 mg of ethidium bromide in 1 000 ml of H_2O.

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 °C.

5.7.5 Solid LB medium, 10 g of bacto $^{TM\ 3)}$-tryptone, 5 g of yeast extract, 5 g of sodium chloride, 15 g of agar, in 1 000 ml of H_2O. After autoclaving for 20 min at 120 °C, LB medium is poured in sterile Petri dishes (20 ml) under a security microbiology post. When solidified, Petri dishes containing LB medium are stored at 4 °C until their use.

5.7.6 Solid LB/Amp medium, is prepared following the recipe of solid LB medium except that after autoclaving for 20 min at 120 °C, 1 ml of ampicilline stock solution at 100 $mg \cdot ml^{-1}$ is added to LB medium.

3) Bacto tryptone is the trademark of a product supplied by Difco Laboratories. 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."