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Second edition
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Soil quality — Direct extraction of soil DNA

Qualité du sol — Extraction directe de l'ADN du sol

Reference number
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Contents

Page

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 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 on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical committee ISO/TC 190/SC 4, *Soil quality,* Subcommittee SC 4 *Biological characterization*

This second edition cancels and replaces the first edition (ISO 11063:2012), which has been technically revised. The main changes compared to the previous edition are as follows (see details in Annex A):

— the amount of soil used (1 g instead of 0,25 g dry mass equivalent);

— the nature and amount of beads (2,5 g of ceramic beads, 2 g of 0,1 mm silica beads and 4 glass beads instead of 0,5 g of 106 µm glass beads and 2 glass beads);

— the duration of the mechanical lysis (90 s instead of 30 s);

— the salt used to precipitate the proteins (potassium acetate instead of sodium acetate).

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 (deoxyribonucleic acid) is an essential component of any living organism coding for enzymes responsible for any biological activities. The study of DNA sequences from DNA sources extracted from different matrices, 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 environments, 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:

a) the extraction of DNA representative of the indigenous bacterial community composition;

b) PCR bias, such as the choice of primers, the concentration of amplified DNA, errors in the PCR, or even the method chosen for analysis.[4],[7],[8],[9] Recently, numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils[10].

The first edition (ISO 11063:2012) described the procedure used to extract DNA directly from soil samples suitable for the study of the composition of microbial community by both quantitative (q-PCR) and qualitative (A-RISA) approaches[11].

The aim of this document is to describe a new method recently reported[12] derived from the first edition procedure to analyse the diversity of soil microorganisms by next-generation sequencing of ribosomal amplicons generated by polymerase chain reactions (PCR) using soil DNA as template. This new method was shown to increase the DNA recovery and to better represents the abundance and the structure of archaeal and fungal communities as compared to the original method[12].

Soil quality — Direct extraction of soil DNA

1 Scope

The present document specifies a method for direct extraction of DNA from soil samples to analyse the abundance and composition of microbial communities by various techniques of molecular biology including real-time quantitative PCR (qPCR). This method is mainly dedicated to agricultural and forest soils. This method can possibly not be suitable for soils rich in organic matter (e.g. peat soils) or soils heavily polluted with organic pollutants or heavy metals.

The direct extraction of DNA from soil samples provides unique insight into the α- and β-diversity of microbial communities. Next-generation sequencing of amplicons obtained by PCR (polymerase chain reaction) amplification of soil DNA constitutes a promising domain which will in the near future contribute to the development of routine tools to monitor microbial communities in soil environments.

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*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
soil DNA
DNA extracted from soil-living microorganisms and remaining DNA from dead microorganisms

4 Principle

DNA is directly extracted from 1 g soil samples (dry weight equivalent) using the following extraction procedure. Soil samples added with an extraction buffer and glass beads are submitted to mechanical and chemical lyses. The lysis step, e.g. by bead beating, is a crucial step to also extract DNA from microbes that are difficult to lyse. Samples are then submitted to chemical lysis by incubation at 70 °C for 30 min. After a brief centrifugation, soil debris are removed, and the supernatant is collected. Part of it is added with potassium acetate to precipitate proteins. After centrifugation, the supernatant is recovered, and nucleic acids are precipitated with ice-cold isopropanol. After centrifugation, the nucleic acids pellet is washed with 70 % ethanol and suspended in sterile ultra-pure water or in TE buffer. DNA quality is then checked by electrophoresis on an agarose gel and the DNA quantity is estimated using a fluorometer. A schematic overview of the procedure is given in Figure 1. Differences between the original (ISO 11063:2012) and this document are listed in a table in the Annex A.

Users of the method ought to be aware that although soil submitted to the DNA extraction procedure is sieved thoroughly (2 mm mesh, procedure described in 5.1), plant residues can still remain in soil samples and, as a result, traces of plant DNA can contaminate the soil DNA extract.

5 Test materials

5.1 Soil

Soil samples should be collected and sieved (2 mm mesh). If samples are not immediately processed, they shall be stored for up to two years at −20 °C or up to 10 years at −80 °C or in liquid nitrogen (−180 °C) as specified in ISO 18400-206. If soil samples are frozen, they may be thawed only once. Some of these storage conditions are currently under testing.

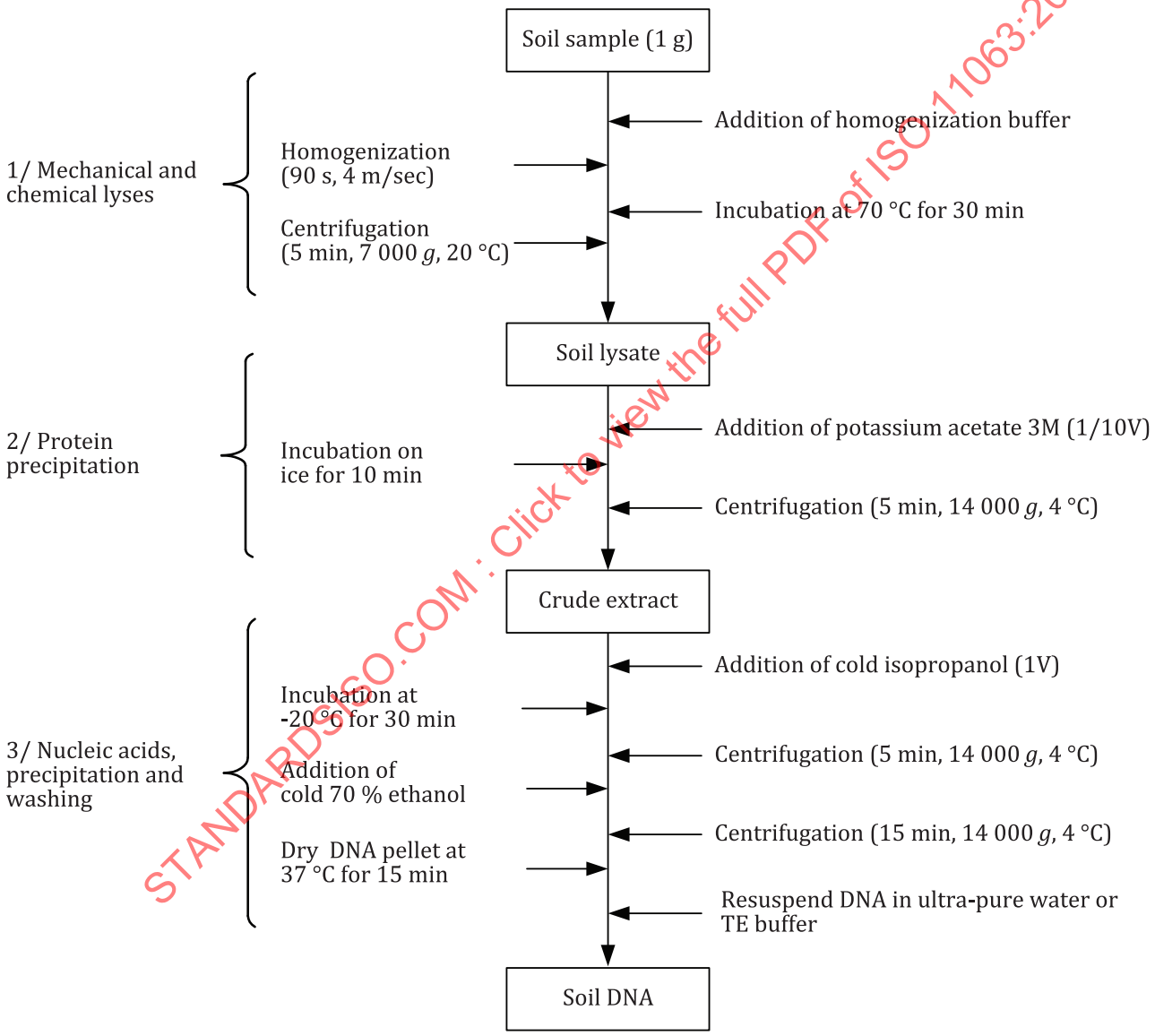

Figure 1 — Schematic overview of soil DNA extraction procedure

5.2 Chemicals

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

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

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

5.2.4 Sodium dodecyl sulfate (SDS), $CH_3(CH_2)_{11}OSO_3Na$ (CAS No. 151-21-3).

5.2.5 Polyvinylpyrrolidone (PVP), $[C_6H_9NO]_n$ (CAS No. 9003-39-8).

5.2.6 Boric acid, $B(OH)_3$(CAS No. 10043-35-3).

5.2.7 Potassium acetate, CH_3COOK (CAS No. 127-08-2).

5.2.8 Acetic acid or glacial acetic acid, CH_3COOH (CAS No. 64-19-7).

5.2.9 Isopropanol, $CH_3CHOHCH_3$ (CAS No. 67-63-0).

5.2.10 Ethanol, CH_3CH_2OH (CAS No. 64-17-5).

5.2.11 Molecular-biology-grade water, H_2O.

5.3 Buffers and reagents

Buffers and reagents (except intercalent molecules, ethanol, isopropanol and SDS) used for soil DNA extraction are prepared with molecular-biology-grade water (5.2.11), sterilized (120 °C for 20 min) and stored at room temperature. Ethanol and isopropanol are stored at −20 °C. When needed pH of buffers and reagents is adjusted using a pH meter.

5.3.1 Tris-HCl, 1 mol/l, 121,14 g of tris in 1 000 ml of H_2O, adjusting with 4 mol/l HCl to pH 8,0.

5.3.2 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.

5.3.3 NaCl, 1 mol/l, 58,44 g of NaCl in 1 000 ml of H_2O.

5.3.4 PVP 40, 200 g/l, 200 g of PVP in 1 000 ml of H_2O.

5.3.5 SDS, 200 g/l, 200 g of SDS in 1 000 ml of H_2O.

5.3.6 Homogenization buffer (newly prepared just before being used), 100 ml of 1 mol/l tris-HCl (pH 8,0), 200 ml of 0,5 mol/l EDTA (pH 8,0), 100 ml of 1 mol/l NaCl, 50 ml of 200 g/l PVP 40, 100 ml of 200 g/l SDS in 450 ml of H_2O.

5.3.7 Potassium acetate, 3 mol/l (pH 5,5), 176.5 g of CH_3COOK in 800 ml of H_2O. Add 100 ml of acetic acid and then adjust the pH to 5,5 with glacial acetic acid (pH measurement recommended). Add water to make up to 1 000 ml.

5.3.8 Ethanol, 700 ml/l, 700 ml of pure ethanol in 300 ml of H_2O.

5.3.9 TE buffer, pH 8,0, 10 mmol/l tris-HCl (5.3.1.), 1 mmol/l EDTA (5.3.2) in H_2O.

5.3.10 Glass beads (4 mm).

5.3.11 Silica beads (0,1 mm).

5.3.12 Ceramic beads (1,4 mm).

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

5.3.14 Fluorescent nucleic acid stain, excitation at 480 nm and emission at 520 nm.

5.3.15 Calf thymus DNA (100 ng/µl).

5.3.16 TBE buffer 10, pH 8,0, 108 g of tris, 55 g of boric acid, 40 ml of 0,5 mol/l EDTA (pH 8,0) in 1 000 ml of H_2O.

5.3.17 TBE buffer ·1, 100 ml of TBE buffer ·10 in 900 ml of H_2O.

6 Apparatus

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

6.1 Homogenizer apparatus, with the ability to program the homogenization speed and duration.

6.2 Fluorometer, allowing the quantification of double-strand DNA at 520 nm emission with a fluorescent nucleic acid stain excited at 480 nm.

7 Procedures

7.1 Preparation of soil samples

Weigh 1 g of soil (equivalent dry mass) in 15 ml tubes just before extracting, or if not processed immediately freeze the soil sample in liquid nitrogen after sampling and keep it frozen at −80 °C until its use.

7.2 Mechanical and chemical lyses

Add 4 glass beads (4 mm in diameter), 2 g of 0,1 mm silica beads (wear a mask for protection) and 2,5 g of ceramic beads (1,4 mm diameter) to the soil sample. Add 5 ml of homogenization buffer (composition given in 5.3.6). Agitate the soil samples at 4 m/s for 90 s using a homogenizer. Incubate at 70 °C for 30 min (preferably in a water bath). Vortex the samples after 15 and 30 min of incubation. Centrifuge for 5 min at 7 000 *g* (20 °C). Carefully recover the supernatant and transfer it to a new 5 ml tube. Collect 1 ml of the supernatant and put it in 1,5 ml tube for protein precipitation. The rest of the lysate can be stored at -20 °C for further DNA re-extraction.

7.3 Protein precipitation

To 1 ml of the supernatant obtained in 7.2, add 3 mol/l potassium acetate (pH 5,5) (composition given in 5.3.7) of an amount that is 1/10 of the volume of the supernatant. Mix by vortexing and incubate on ice for 10 min. Centrifuge for 5 min at 14 000 *g* (4 °C). Carefully recover the supernatant and transfer it to a new 2 ml tube.

7.4 Nucleic acid precipitation and washing

Perform all these steps below a fume hood because of dangerous isopropanol vapours. Liquid and solid wastes shall be evacuated as chemical waste.

To the supernatant obtained in 7.3, add cold isopropanol (-20 °C) of an amount that is 1/1 of the volume of the supernatant. Incubate the samples at −20 °C for 30 min. Centrifuge for 30 min at 14 000 *g* (4 °C). Carefully eliminate the supernatant. Wash the nucleic acids pellet with cold 700 ml/l ethanol (do not resuspend the pellet). Centrifuge for 5 min at 14 000 *g* (4 °C). Eliminate any traces of ethanol and let the nucleic acid pellet dry for 15 min at 37 °C. Suspend the pellet in 200 µl of ultra-pure water or TE buffer (pH 8) (composition given in 5.3.9).

7.5 Nucleic acid storage

Aliquot the soil DNA (4,50 µl) and store the DNA samples at −20 °C until their use. Repeated freezing and thawing of the DNA extracts should be omitted.

8 Estimation of soil DNA quality and quantity

8.1 Soil DNA quality and purity

The quality and the size of the soil DNA are checked by electrophoresis on 1 % agarose gels in TBE buffer. Gels are stained with appropriate staining (e.g. ethidium bromide, 5 mg/l). The purity of the soil DNA is assessed by spectrophotometry at 260 nm for the DNA analysis and at 340 nm for humic acid substances[11].

The step of chemical and mechanical lysis is critical, and it should be adequate to lyse a representative portion of microbes but avoid fragmentation of the DNA[14].

DNA extracts which are still slightly coloured need a further DNA purification (see Annex B for possible methods to further purify soil DNA extracts).

8.2 Soil DNA quantity

The soil DNA content is determined using a fluorescent nucleic acid stain (5.3.14) which fluoresces when intercalated within the double helix of DNA. A calibration curve relating the amount of standard DNA (5 ng, 10 ng, 20 ng, 50 ng, 100 ng, 150 ng and 200 ng of calf thymus DNA) to the amount of fluorescence quantified is established and used to estimate the amount of DNA extracted from the soil. Measurements are performed using a fluorometer (6.2). The analysis is carried out by relevant software.

Alternatively, the soil DNA content can be determined by resolving soil DNA extracts by electrophoresis in a 1 % agarose gel, stained with ethidium bromide and photographed under a camera. Dilutions of calf thymus DNA were included in each gel and a standard curve of DNA concentration (1 000 ng, 500 ng, 250 ng, 125 ng, 62,5 ng to 31,25 ng). The ethidium bromide intensity was integrated to establish a standard curve used for estimating soil DNA concentration as described previously by Reference [15].

Alternatively, the soil DNA content can be determined by spectrophotometry at 260 nm when soil DNA is lowly contaminated with humic acid substances (340 nm) and proteins (A260/A280 averaging 1,6)[16].

9 Validation of the extraction procedure

The laboratory can validate the procedure of soil DNA extraction by processing a reference soil sample stored under appropriate conditions (as specified in ISO 18400-206) and comparing the obtained yield of soil DNA extraction to the expected one.

NOTE Alternatively artificial mixtures of different microbes (including different bacterial and fungal species) can be used to prove quality of soil DNA extraction. Such standards are under development and validation and will be implemented to that guideline at later stage.

10 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 11063:2020;

b) soil collection, including date and place (GPS coordinates) of collection;

c) treatment and storage of soil sample (e.g. sieving method, conditions and length of storage);

d) physical and chemical characteristics of the soil;

e) date(s) of extraction;

f) homogenizer used for mechanical lysis;

g) duration of nucleic acids storage (if appropriate);

h) tables of results including concentration of soil DNA extracts and amount of DNA extracted per gram of soil (dry weight equivalent);

i) any details not specified in this International Standard or which are optional, as well as any effect which may have affected the results.

Annex A
(informative)

Differences between ISO 11063:2012 and the revised document for direct extraction of DNA from soil samples

Table A.1 below presents step by step the differences between the method of the first edition of ISO 11063 (i.e. ISO 11063:2012) and the method of this edition (i.e. ISO 11063:2020) for direct extraction of DNA from soil samples.

Table A.1 — Differences between ISO 11063:2012 and the revised document for direct extraction of DNA from soil samples

Step	ISO 11063:2012	ISO 11063:2020
Soil sample	0,25 g	1 g
Bead-beating	1 600 *g*, 30 s	4 m/s, 90 s
Incubation	70 °C, 10 min	70 °C, 30 min
Centrifugation	14 000 *g*, 1 min, 4 °C	7 000 *g*, 5 min, 20 °C
Protein precipitation	sodium acetate 5M (1/10V)	potassium acetate 3M (1/10V)
Nucleic acids precipitation	-20 °C, 15 min	-20 °C, 30 min
1st centrifugation	14 000 *g*, 30 min, 4 °C	14 000 *g*, 5 min, 4 °C
2nd centrifugation	14 000 *g*, 15 min, 4 °C	14 000 *g*, 15 min, 4 °C

Annex B
(informative)

Possible methods to purify soil DNA extracts

As mentioned in 8.1, soil DNA extracts which are still coloured* need a further purification to retrieved humic acid substances which may interfere with Taq-polymerase. Several options can be followed to purify soil DNA extracts.

Recommendations may include the combined use of PVPP- and Sepharose®[1)] 4B-columns as done in the inter-laboratory evaluation of the ISO standard 11063 (2012)[11]. The PVPP-column is an affinity column retaining humic acid substances while Sepharose 4B-column is an exclusion column desalting DNA extracts.

It may also be recommended to apply more recent purification systems relying on the use of silica membrane columns or silica coated magnetic beads. For both silica-based systems the principle of the purification relies on the binding of DNA to silica that is washed several times to remove contaminants prior to DNA elution.

*Humic acid concentration in soil DNA extracts can be calculated from absorbance measurements at 340 nm using a calibration curves with serial dilutions (0,1 – 100 $ng.\mu l^{-1}$) of commercial humic acids[16].

1) Sepharose is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.