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High-Throughput Isolation of Nucleic Acids from Soil

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Abstract: DNA-based technologies have become widespread tools for soil microbiological analyses in recent years. DNA extraction from the soil is a key step for these approaches: it is a challenge for researchers as it is still both expensive and time-consuming when large surveys are planned. The aim of this study was to develop a high-throughput automated protocol for DNA extraction and purification from soil. The protocol was based on the BioSprint 96 platform and compared for validation with another automated procedure and two commercial column-based kits. To evaluate the performances of the protocols, we considered quality, quantity, and amplifiability of the isolated DNA. The material isolated by means of the four protocols showed appropriate yield and quality and positive amplification. The isolation protocol presented here provided similar results to those of the commercial kits but with two essential differences: cost and time for DNA extraction were drastically reduced. This rapid and efficient protocol is envisaged as ideal to standardize soil studies and treat large numbers of samples, representing a workable alternative to low-throughput and expensive manual extraction methods.

Keywords: soil; DNA extraction; automated method; DNA quantity, purity and amplifiability

1. Introduction

Soil is a very complex environment containing huge microbial diversity [1,2]. Its characteristics depend on physical and chemical but also biological factors [3]. The biotic component forms up to approximately 0.2% of the soil, with microorganisms representing just 20–40% and controlling 80-90% of soil processes [4,5]. Biodiversity in soil is enormous, composed of both micro- and mesoorganisms, but the processes in which these are involved are still barely known [6]. Given our poor knowledge concerning the role of the biotic fraction in soil biochemical processes, a deeper understanding of soil biodiversity and its functions is greatly needed.

The majority of soil microorganisms cannot be cultivated and characterized by conventional laboratory methods [7,8]. Culture-independent methods are therefore required for their study and DNA-based technologies have been developed over the years to bypass the limits of microorganisms cultivation.

The objective of these approaches is to achieve good-quality DNA, since successful downstream analyses mostly depend on this. According to Bessetti et al. and Huang et al. [9,10] soil contains a large

amount of inhibitory compounds that prevent or interfere with DNA amplification. These substances are the major obstacle to the following PCR amplification steps. Inhibitors can co-precipitate with DNA, adversely affecting the quality and quantity of the extract [11,12]. Among inhibitory substances, humic compounds are the most common, followed by heavy metals and aromatic compounds. Fornasier et al. [13] define DNA extracted without any purification process as "crude DNA", which usually presents a relatively high yield but low amplifiability because of its content of inhibitors. Thus, a purification step after DNA extraction and/or the use of diluted DNA is recommended for PCR applications [14].

Several manual extraction kits for soil DNA have been developed so far, aiming to guarantee high DNA quantity, purity, and amplifiability. These kits have been extensively validated and many studies report comparisons to identify the most appropriate one for each aim [15–18]. Most of them combine DNA extraction and purification, while others only perform the purification step. In both cases, the extraction process starts with cell lysis by bead beating, the most efficient way to extract nucleic acids from cells [19], followed by a purification step by means of silica columns. Moreover, although many methods with different advantages and disadvantages have been developed [20,21], none of them allows a satisfactory standardization of the procedure, nor its amenability to conveniently handle large numbers of samples, which are the main drawbacks in all manual protocols. However, land-wide surveys and soil microbiome consortium studies adopting reproducibly standardized protocols and high processivity are becoming the rule (http://www.earthmicrobiome.org/). This is why automated high- and medium-throughput solutions for DNA extraction and purification appear to be appropriate options to conduct efficient global campaigns of soil analyses and achieve results comparable with those of parallel studies. It is thus clear that the current research in soil microbiology would greatly benefit from an automated protocol for DNA isolation that could standardize reports in this field.

The aim of this study was to develop an automated high-throughput protocol for DNA isolation from soil. To validate our protocol, we tested yield, purity, and amplifiability of the isolated DNA. We compared these parameters against those obtained from DNA isolated with another automated protocol and two manual kits.

2. Results

Information about the chemical-physical properties of the soils analyzed is reported in the Materials and Methods section, together with a description of the isolation methods considered in this work. DNA quantity and purity were measured after DNA isolation with the four protocols as well as their amplifiability.

2.1. DNA Quantity, Purity, and Amplifiability

Yield (μg/g) and purity (A_{260}/A_{280}) obtained by the preliminary "crude DNA" step and by each of the four isolation methods for the different soils are shown (Table 1). Data are the mean of three biological replicates.

The "crude DNA" method resulted as expected in the highest yield of spectrophotometrically-recordable absorbance at 260 nm, followed by the PowerSoil DNA Isolation Kit. We then obtained similar results using the BioSprint 96, QIASymphony, and FastDNA SPIN Kit protocols. The difference between the quantity of DNA obtained from the four methods and the "crude DNA" section of the protocol was highly significant ($p < 0.01$). Other highly significant differences were observed in the DNA yield of BioSprint 96, QIASymphony, and FastDNA and PowerSoil isolation protocols ($p < 0.01$). No statistically significant differences were observed instead in the DNA yield among the three soils. This (horizontal) comparison is not shown.

Table 1. The upper half of the table reports the assessment of yield and purity of DNA obtained by the four isolation methods, for the three soils (S1, S2, S3). Standard errors are also shown. Means with the same letter in the (vertical) comparison among protocols are not significantly different from one another at Duncan's test. The bottom half of the table reports the gene copies number obtained by the four isolation methods, for the three soils (S1, S2, S3), with standard errors. The table reports the two targets considered in the study (Arch-*amoA*, *nosZ*). Values are calculated basing on the mean Ct values. Values with the same letter in the (vertical) comparison among protocols are not significantly different from one another at Duncan's test.

	Method	S1	S2	S3
Yield (µg/g)	"Crude DNA"	4.742 ± 0.092 a	3.005 ± 0.015 a	4.217 ± 0.044 a
	BioSprint 96	0.132 ± 0.003 c	0.079 ± 0.002 c	0.095 ± 0.004 d
	QIASymphony	0.140 ± 0.012 c	0.083 ± 0.017 c	0.101 ± 0.023 c
	FastDNA	$0.051 \pm 3 \times 10^{-4}$ d	$0.095 \pm 6 \times 10^{-4}$ c	$0.109 \pm 6 \times 10^{-4}$ c
	PowerSoil	0.366 ± 0.002 b	0.178 ± 0.001 b	0.280 ± 0.001 b
Purity (A_{260}/A_{280})	"Crude DNA"	1.24 ± 0.005 c	1.28 ± 0.006 c	1.25 ± 0.005 c
	BioSprint 96	1.80 ± 0.003 b	1.84 ± 0.008 a	1.35 ± 0.021 b
	QIASymphony	1.84 ± 0.011 b	1.85 ± 0.015 a	1.42 ± 0.013 b
	FastDNA	1.71 ± 0.003 b	1.49 ± 0.006 b	1.69 ± 0.003 b
	PowerSoil	2.11 ± 0.003 a	1.70 ± 0.006 b	1.75 ± 0.003 a
Gene copies Arch-*amoA*	"Crude DNA"	$6.80 \times 10^{1} \pm 1.33 \times 10^{1}$ d	$3.84 \times 10^{2} \pm 8.52$ d	$4.41 \times 10^{2} \pm 3.90 \times 10^{1}$ d
	BioSprint 96	$1.46 \times 10^{6} \pm 3.84 \times 10^{5}$ b	$6.36 \times 10^{5} \pm 9.70 \times 10^{4}$ c	$2.44 \times 10^{6} \pm 4.40 \times 10^{5}$ c
	QIASymphony	$3.44 \times 10^{6} \pm 7.04 \times 10^{5}$ b	$8.43 \times 10^{5} \pm 5.16 \times 10^{4}$ c	$2.84 \times 10^{6} \pm 5.25 \times 10^{5}$ c
	FastDNA	$1.79 \times 10^{5} \pm 3.82 \times 10^{4}$ c	$1.80 \times 10^{6} \pm 2.06 \times 10^{4}$ b	$4.70 \times 10^{6} \pm 1.04 \times 10^{6}$ b
	PowerSoil	$1.64 \times 10^{7} \pm 1.14 \times 10^{6}$ a	$1.56 \times 10^{7} \pm 1.82 \times 10^{6}$ a	$2.04 \times 10^{7} \pm 3.51 \times 10^{5}$ a
Gene copies *nosZ*	"Crude DNA"	$1.88 \times 10^{6} \pm 2.16 \times 10^{5}$ d	$1.29 \times 10^{6} \pm 2.35 \times 10^{4}$ d	$1.59 \times 10^{6} \pm 4.62 \times 10^{3}$ d
	BioSprint 96	$1.45 \times 10^{7} \pm 8.64 \times 10^{5}$ b	$3.79 \times 10^{6} \pm 8.85 \times 10^{4}$ c	$2.97 \times 10^{7} \pm 1.13 \times 10^{6}$ b
	QIASymphony	$1.54 \times 10^{7} \pm 1.40 \times 10^{6}$ b	$3.86 \times 10^{6} \pm 5.65 \times 10^{5}$ c	$2.59 \times 10^{6} \pm 1.32 \times 10^{6}$ c
	FastDNA	$5.39 \times 10^{6} \pm 1.43 \times 10^{5}$ c	$1.27 \times 10^{7} \pm 1.64 \times 10^{6}$ b	$2.10 \times 10^{7} \pm 3.54 \times 10^{6}$ b
	PowerSoil	$8.18 \times 10^{7} \pm 7.78 \times 10^{6}$ a	$2.82 \times 10^{7} \pm 7.17 \times 10^{5}$ a	$9.04 \times 10^{7} \pm 1.10 \times 10^{7}$ a

The "crude DNA" method resulted in the lowest (A_{260}/A_{280}) ratio ($p < 0.01$), followed by the FastDNA SPIN Kit for Soil, the automated methods, and the PowerSoil DNA Isolation Kit. The statistically significant differences in the level of purity of DNA isolated with the four protocols ($p < 0.01$) are indicated by different letters. All of them showed a major difference when compared to the "crude DNA" preparation. No statistically significant differences were observed in the purity among the three soils.

We also report the gene copies number for both the Arch-*amoA* and *nosZ* targets (Table 1). Information about the PCR conditions is reported in Materials and Methods, as well as primer sequences (Table 5). These data were obtained by means of equations (Table 6) using thermal cycles (Ct) of amplification (see Supplementary Materials for raw Ct data). Each Ct resulted from the mean among three biological replicates, for each protocol and each soil sample. For both the Arch-*amoA* and *nosZ* targets, the highest Ct values (lowest gene copies number) were observed (i.e., the lowest deducible quantity of target genes) when the "crude DNA" material was used as a straight template source, suggesting the presence of inhibitors.

2.2. Consumable Cost and Time Per Sample

The automated protocol based on BioSprint 96 is about five times cheaper than the PowerSoil DNA Isolation Kit and the FastDNA SPIN Kit for Soil, while it is three times cheaper than the protocol based on QIASymphony (Table 2).

Our automated protocol based on BioSprint 96 requires about 1 h for the extraction of 96 samples, whereas the QIASymphony protocol takes around 4 h (but with the possibility to run overnight) and the two manual protocols require about 8 h (Table 2).

Table 2. Cost per sample and processing time required for DNA isolation from 96 soil samples.

Method	Cost (€/Sample)	Processing Time (96 Samples)
BioSprint 96	1.50	0.3 h run + 0.5 h samples prep (50 min) *
QIASymphony	5.00	3 h run + 1 h samples prep (4 h) **
FastDNA	8.00	8 h
PowerSoil	7.50	8 h

* The run process being automatized, 20 min out of 50 are labor-free; ** The run process being automatized, 3 h out of 4 are labor-free.

3. Discussion

In our study, we compared two automated protocols and two manual kits for DNA isolation from soil samples. The automated procedures were not originally designed for DNA isolation from soil: the BioSprint 96 protocol was developed to isolate DNA from plants, while the one for QIASymphony was modified from the protocol to isolate DNA from blood and possibly other substrates rich in inhibitors. Although they were not protocols purposely recommended for soil DNA, both automated methods provided suitable results, comparable to those obtained from the two manual kits. The four protocols all provided extracts whose yield and purity were within workable ranges and devoid of major inhibiting effects on DNA amplifiability.

As regards to the "crude DNA", it yielded a conspicuous quantity of A_{260}-adsorbing material. Such a reading is to be considered as the effect of a series of other compounds, including proteins and several soluble organics from the soil, which have absorbance spectra that amply overlap with the maxima displayed by nucleic acids. The overall yield of the "crude DNA" is therefore in large part to be considered only as an apparent decrease in DNA content, which was instead overestimated by the crude reading.

The low purity of the "crude DNA" is testified to the low A_{260}/A_{280} ratio (Table 1) which, in sufficiently pure preparations, is commonly expected to be above 1.7 [11]. Such a value, representing an efficient purification from the protein components, is substantially observed in the four protocols tested.

The analysis of DNA amplifiability confirmed, as expected, a strong dependence on its quality, indeed all the analyzed methods presented better amplification (low Ct) when the DNA was of better quality (high A_{260}/A_{280} ratio).

Interestingly, DNA yield and amplifiability reflect the organic matter (OM) quantity contained in the soil sample. The sample with the highest level of organic carbon was S1, followed by S2, and S3 (Table 3). S1 gave the highest DNA yield but the sample with the highest amplifiability was S3, for both targets. While higher organic matter contents could be linked to higher DNA content, the lower the organic matter is, the lower the content of PCR inhibitors would be. This means that the soil features play a key role in the quantity of DNA that can be isolated and amplified, but also, that each experiment must be carefully calibrated on those characteristics. These results are in agreement with a similar study by Martoz et al. [17], who compared the performances of automated protocols and manual kits in the extraction of DNA from different substrates. They also found "that any effect of extraction method on sequencing results was small compared with the variability across samples". We thus suggest an earlier characterization of the samples for a more reliable interpretation of the results.

Table 3. Physical-chemical properties of the soils (dry weight).

Sample ID	Land Use	Sand (%)	Silt (%)	Clay (%)	pH (H_2O)	OM (g kg^{-1})
S1	Pasture	94	2	4	7.45	3.8
S2	Vineyard	44	16	40	7.8	2.2
S3	Arable	66	10	24	7.5	1.7

The soils that we analyzed in this study had a low organic matter content, but they were chosen as representative of commonly encountered ranges in agricultural soils. It would be interesting to

analyze samples with higher amounts of OM, such as e.g., forest soils, to further assess the correlation between organic matter content and inhibitors and whether a trade-off maintaining amplifiability in spite of sample dilution and purification could be defined. Further analysis could also consider more targets: Arch-*amoA* and *nosZ* genes are commonly used as functional markers given their importance in nitrogen-related processes [22], but more sequence targets are available to cover further examples across soil biodiversity. However, the two chosen genes were selected because they target very different taxa, being the nitrifying autotrophic ammonia oxidizers in the *Archaea* domain, while the *nosZ* bearing cells well represent heterotrophic eubacteria. These two functional groups not only encompass many species but also two very different cell types in terms of morphology, occupied microniches within the soil structure and consequent response to the lysis step of the protocols. The fact that we observed prompt amplification responses for both genes can be considered a positive check of the overall protocol efficiency in reaching different targets.

Given that the four isolation protocols provided satisfactory and essentially comparable results, the most significant differences among them were not in terms of yield, purity or amplifiability, but on the side of cost and time. The protocol based on the BioSprint 96 allows isolation at 20% the cost of the manual kits and 30% the cost of the QIASymphony. Moreover, the BioSprint 96 protocol allows DNA to be isolated from 96 soil samples in less than 1 h by one person (less than one minute per sample), whereas the other three methods would take a whole day's work (or several operators and sets of different centrifuges, etc.). To make another comparison, Miao et al. [18] developed what was considered as a rapid extraction protocol in two steps, which allows the extraction of each sample in 70 min. Orgiazzi et al. [23] report the tendency to move from PCR-based methods targeting a single DNA fragment to those based on the sequencing of the whole genome in the soil. Metagenomic approaches for such a vast and heterogeneous environment as soil require a large number of samples to be representative of the whole biodiversity. DNA extraction and purification from so many samples is not feasible with manual kits, as it is costly and time-consuming. If we consider the rise of metagenomics techniques and the global joint efforts of research consortia needing common protocols for the comparability of results, an alternative to column-based manual kits for DNA extraction looks even more urgent. The scientific community is indeed already asking for a high-throughput method for the isolation of DNA from soil samples. Martoz et al. [17] asserted that a rapid and efficient DNA extraction represents the major bottleneck for metagenomic sequencing: "As microbiome analyses become applicable to an increasing number of scientific areas, a streamlined process for efficiently extracting DNA to generate 16S rRNA gene amplicons or shotgun metagenomic sequencing data from a range of environmental sample types is increasingly important".

Surprisingly, a protocol developed for plants with appropriate modifications performed similarly to methods conceived specifically for soil but was faster and cheaper. Given the need to standardize the study pipelines and overcome biases derived from manual methods [23–25], an automated method qualifies as a promising strategy for unified and concerted programs to study the global diversity of our soils.

4. Material and Methods

4.1. Sampling and Chemical-Physical Analysis of Soil

Samples were collected in three different sites of Italy: Belluno (S1), Piove di Sacco (Venice) (S2), and Spinea (Venice) (S3). In each site, 16 sub-samples were collected within an area of 0.5 km^2 at 20 cm depth by means of an Edelman 36-mm diameter manual auger (Eijkelkamp Agrisearch Equipment, The Netherlands). The sub-samples from each site were mixed together to obtain three main samples. Final soil samples were dried at room temperature for 48 h, crushed and sieved (Ø 0.5 mm).

Chemical and physical properties of soil samples were determined as described by Foesel et al. [26] (Table 3).

4.2. DNA Extraction and Purification

0.400 g of each soil was used for each extraction method:

- Automated method 1: The protocol for the BioSprint 96 (Qiagen, Germany) developed for purification of DNA from plant tissue was appropriately modified to perform DNA extraction from soil. Each of the three samples was placed in a 2 mL sterile Eppendorf safe lock microtube (Eppendorf, Germany) with two different sizes of silica beads: 0.4 mL of beads (Ø 0.1 mm) and 0.4 mL of beads (Ø 0.6 mm). 1.2 mL of Na_2HPO_4 extracting buffer (0.12 M, pH 8) was added to the microtubes. Microtubes were loaded in a TissueLyser II (Qiagen) and homogenized for 5 min (30 Hz) for cell lysis. Lysates were centrifuged (20.000 g × 5 min) and the resulting supernatant ("crude DNA") was transferred into 1.5 mL sterile microtubes for purification.

Five 96-deep-well plates (S-Blocks, Qiagen) and one 96-well plate (Microplates MP, Qiagen) were loaded in the BioSprint 96 robotic station for "crude DNA" purification and filled as shown in Table 4.

Table 4. Plate loading for the automated protocol based on BioSprint 96.

Plate Nr.	Plate Type	Volume Per Well (µL)
1	S-Blocks	200 µL supernatant + 200 µL RLT * + 200 µL isopropanol + 25 µL MagAttract Suspension G (Qiagen)
2	S-Blocks	500 µL RPW **
3	S-Blocks	500 µL 96% ethanol
4	S-Blocks	500 µL 96% ethanol
5	S-Blocks	500 µL 0.02 % (*v*/*v*) of TWEEN 20 (Amresco, USA)
6	MP	100 µL PCR-grade H_2O (elution plate)

* Guanidine thiocyanate buffer under patent protection. ** Guanidine hydrochloride buffer under patent protection.

- Automated method 2: the QIASymphony DSP DNA Mini Kit 192–version 1 (Qiagen) was used to extract DNA by means of the QIASymphony platform according to the manufacturer's instructions. No modifications were made to the original protocol, recommended for DNA extraction from blood. The producer suggested this kit as the most appropriate to try DNA extraction from soil.
- Manual method 1: the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) was used to extract DNA according to the manufacturer's instructions. No modifications were made to the original protocol.
- Manual method 2: the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA USA) was used to extract DNA according to the manufacturer's instructions. No modifications were made to the original protocol.

Samples were extracted by means of each method (3 biological replicates for each soil), including 9 samples of "crude DNA". After extraction and purification, the eluted DNA and the "crude DNA" were conserved at −80 °C.

4.3. Evaluation of DNA Quantity, Purity, and Amplifiability

The quantity of double-stranded DNA (dsDNA) extracted from the soils by the different methods was assessed using a Qubit 3.0 fluorometer (Waltham, MA, USA). Before the analysis, a two-point calibration curve was established using the standards supplied with the kit, at 0 ng/µL and 10 ng/µL. According to the manufacturer's instructions, 189 µL of BR buffer, 1 µL of BR reagent (Qubit dsDNA BR Assay Kit, Thermo Fisher Scientific), and 10 µL of the purified sample were placed in clear plastic 0.5 mL Qubit Assay Tubes (Thermo Fisher Scientific). Fluorescence was evaluated at 530 nm. DNA concentration derived from the Qubit measurements and volume of the DNA extract was used to calculate DNA yield with a simple multiplication.

The purity of DNA extracted from the different soils by each method was evaluated using a UV/Vis Biophotometer (Eppendorf). The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) was

used to assess protein contamination. 95 µL of sterile water and 5 µL of the purified sample were placed in disposable UVette cuvettes (Eppendorf) for the analysis.

A real-time PCR targeting the archaeal *amoA* and bacterial *nosZ* genes was used to evaluate the amplifiability of the extracted and purified DNA. qPCR reactions were conducted in 5 µl volumes on the QuantStudio 12K-flex (Life Technologies, Carlsbad, CA, USA) using 384-well plates. The reaction mix was composed of 0.15 µL each of F and R primer (Table 5), 2.5 µL Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA), 1.2 µL PCR-grade water, and 1 µl template DNA. Cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C 15 s; 57 °C 60 s; 72 °C 15 s), 95 °C 15 s, 60 °C 60 s, 95 °C 15 s. This thermal cycle was used for both targets. All reactions were run in triplicate and positive and negative controls were included.

Ct values were used to assess the amount of amplifiable DNA. The Ct value indicates the number of thermal cycles necessary for the emission of the fluorescence signal from the sample. Upon prior insurance that melt curves are compliant with proper amplification, high Ct value means a high number of PCR cycles and thus, low amplifiability. Low Ct values are desirable since they are associated with larger amounts of amplifiable DNA [27].

Table 5. Primer sequences and amplicon lengths.

Primer.	Sequence	Amplicon Length	Reference
Arch-*amoA*-F	5′-STA ATG GTC TGG CTT AGA CG-3′	635 bp	[28]
Arch-*amoA*-R	5′-GCG GCC ATC CAT CTG TAT GT-3′		
NosZ-F	5′-CGY TGT TCM TCG ACA GCC AG-3′	706 bp	[29]
NosZ-R	5′-CAT GTG CAG NGC RTG GCA GA-3‰		

A standard curve using known amounts of the target genes cloned in plasmids of known length was obtained and data were used to calculate the copy number of the gene targets based on the Ct value. As templates for the standard curves, we used purified plasmids in which we had cloned amplicons of each of the target genes into vector pGem-T (Promega Corp., Madison, WI, USA) inserted into *E. coli* JM101 by electroporation. Knowing the size of the vector (3015 bp) and that of each insert from literature references of each primer pair, upon measuring the plasmid DNA concentration, we calculated the number of copies per ng of DNA and the corresponding amounts to be used for each of the quantitative PCR calibration curves. The two interpolative fitting equations were chosen for the two targets on the basis of their highest R2 values (Table 6).

Table 6. Equations used to calculate gene copy number for both the targets, with the respective R^2 values.

Target	Equation	R^2
Arch-*amoA*	$y = 1E + 35x^{-21.52}$	0.9783
NosZ	$y = 2E + 11e^{-0.631x}$	0.9878

4.4. Statistical Analysis

Statistical data analysis was performed using Statistica v. 13.0 (Dell, Round Rock, TX, USA). Data are expressed as mean ± standard error of the mean. Significant differences among the mean values were evaluated with one-way ANOVA followed by post hoc analysis (Duncan's test). Significance was estimated at the $p < 0.01$ level.

5. Conclusions

In conclusion, each protocol provided results compliant with the assessment of the target genes presence and abundance in soil but the high-throughput automated method for DNA extraction from soil can significantly reduce costs and time while keeping performances within the same orders of magnitude and with acceptable tradeoffs in terms of yield, purity, and amplifiability in comparison to

those of common manual kits. Such a protocol is envisaged as a suitable choice to standardize the ever-increasing approaches in soil and environmental studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2571-8789/4/1/3/s1.

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