Soil DNA Purification and Isolation

Hamid kheyrodin*

Semnan University, Iran

Abstract: There is an increased interest in the extraction of nucleic acids from various environmental samples, since molecular techniques allow less biased access to a greater portion of uncultivable microorganisms. Two strategies have been developed to improve DNA recovery in terms of yield, purity and unbiased representation of the microbial diversity. Amplification of DNA from soil is often inhibited by co-purified contaminants. DNA is also suitable for PCR amplification using various DNA targets. This review presents an overview of the available methods to achieve this challenging objective. DNA was extracted from 100g of soil using direct lysis with glass beads and SDS followed by potassium acetate precipitation, polyethylene glycol precipitation, phenol extraction and isopropanol precipitation.

Keywords: PCR, soil, DNA, RNA.

1. INTRODUCTION

The inability to culture most microorganisms from environmental samples is a fundamental obstacle to understanding microbial ecology and diversity [2]. The use of DNA-based techniques can overcome this limitation by allowing the fate of particular genes or organisms to be monitored directly in environmental samples. Techniques to extract DNA from soil and sediment initially used large samples of 100g [3, 4]. These extracts were usually contaminated with humic acids which interfered with subsequent molecular biological manipulations. Extensive purification steps were then required to successfully amplify a PCR product, including CsCl-ethidium bromide density gradient centrifugation [4-6], or the use of commercial reagents [7-11]. These steps increase both the complexity and the cost of the technique. This paper describes in detail a method for extracting DNA from soil which involves minimal purification prior to PCR amplification [1]. The method is compared to other commonly used DNA extraction methods. A PCR product was obtained rapidly and inexpensively from large amounts of soil, even when contaminated with heavy metals. A rapid, inexpensive, large-scale DNA extraction method involving minimal purification has been developed that is applicable to various soil types [1].

2. MATERIALS AND METHODS

2.1. Soil DNA Purification Protocol

2.1.1. A. Preparation

Spin Columns1. Add 550µl of Inhibitor Removal Resin to each empty Spin Column to be used. Centrifuge for 1 minute at 2000 x g to pack the column. 2 Decant flow-through and place the column in the same collection tube. 3 Add another 550µl of Inhibitor Removal Resin to each packed column. Centrifuge for 2 minutes at 2000 x g. 4 Move the column to a clean 1.5-ml collection tube.

Pellet Wash Solution: 1 For 50 Extractions Kit: Add 45ml of ethanol to the Pellet Wash Solution before first use. For 5 Extractions Kit: Add 4.5ml of ethanol to the Pellet Wash Solution before first use.

B. Cell Lysis: 1 Weigh out 100mg of the soil sample into a 1.5ml tube. 2 Add 250µl of Soil DNA Extraction Buffer and 2µl of Proteinase K; vortex briefly. 3 (Optional) To increase the yield of DNA, shake the tube at 37°C for 10 minutes or vortex for 2 minutes. Note: vortexing may shear the DNA. 4 Add 50µl of Soil Lysis Buffer and vortex briefly. 5 Incubate at 65°C for 10 minutes. 6 Centrifuge for 2 minutes at 1000 x g 7. Transfer 180µl of the supernatant to a new tube 8. Add 60µl of Protein Precipitation Reagent, mix thoroughly by inverting the tube 9. Incubate on ice for 8 minutes. Centrifuge the tube for 8 minutes at maximum speed 10. Carefully transfer 100-150µl of the supernatant directly onto the prepared Spin Column (from Section A). 11 Centrifuge for 2minutes at 2000 x g into the 1.5-ml tube. Discard the column. 12 Add 6µl of DNA Precipitation Solution, vortex briefly. Incubate the tube at room temperature for 5minutes. 13 Centrifuge for 5 minutes at maximum speed. Carefully decant the supernatant. 14 Wash the pellet with 500µl of Pellet Wash Solution (prepared in Section A). Invert to mix then spin for 3 minutes at maximum speed. Carefully decant the supernatant. 15 Repeat the wash and spin16. Resuspend the pellet in 300µl of TE Buffer.

2.2. Troubleshooting DNA Extractions

DNA does not amplify by PCR1) Optimize cycling conditions. Decrease the annealing temperature of the

*Address correspondence to this author at the Semnan university, Iran; Tel: 023 33335405; Fax: 023 33335404; E-mail: kkhyrodin@gmail.com

E-ISSN: 2409-9813/15

cycling profile by 2 degrees or more. Some primer pairs require a lower annealing temperature (less stringent conditions) when amplifying soil DNA. (2) Use less starting material. Some environmental samples contain significantly larger amounts of enzymatic inhibitors. When using these samples, begin the extraction with less starting material (50mg). (3) Load less extract onto the column. If any color remains in the extract after the Inhibitor Removal Spin Column step, load less extract onto the column. (4) Dilute the extracted DNA. Dilute the extracted DNA 2-10 fold before

(5) Rewash the pellet with the Pellet Wash Solution. This step is important in removing residual inhibitors of DNA amplification. DNA is sheared. (1) Eliminate the vortex mixing step. Eliminate the 2minute vortex mixing step when extracting the DNA. Shake at 37°C instead or simply skip this step entirely.

Soil (loamy sand) was collected on campus at semnan university in Iran. The sokan semnan National Park Station samples represent the extremes of pristine vs polluted soils and were compared by further soil testing (Table **1**).

Table 1: Analysis of Soil Samples

pH	3.90	6.93
Organic matter (%)	5.09	16.3
Field capacity 0.33 Bar	7.05	14.9
CEC (cmol (+)/kg)*	1.1	18.7
As (mg/kg)#	< 3	6.9
Hg(mg/kg)#	< 0.7	2.1
Zn(mg/kg)#	5	1818
Cr(mg/kg)#	3.3	30.4
Cd(mg/kg)#	< 0.4	11.4
Ni(mg/kg)#	1.7	98.3
Pb(mg/kg)#	15	520
Cu(mg/kg)#	9.5	268
Mn(mg/kg)#	13	518

2.3. DNA Extraction Using Bead Beating (1)

Extraction buffer (100ml of 100mM Tris-HCl [pH 8.0], 100mM sodium EDTA [pH 8.0], 1.5M NaCl) was mixed with 100g (wet weight) of soil. Glass beads (100g, Bio-Spec Products, Bartesville, U.S.) were added and the sample blended in a Bead-Beater (Bio-Spec Products) for 2 minutes. Sodium dodecyl sulphate (SDS) was added (10ml; 20%) and blending continued for a further 5 sec. The sample was incubated at 65°C for 1hr, transferred to centrifuge bottles (250ml) and centrifuged at 6000g for 10 min. The supernatant was collected, and the soil pellet re-extracted with further extraction buffer (100ml), incubation at 65°C for 10 minutes and centrifugation as above. Supernatants were transferred to centrifuge tubes (50ml) containing a half-volume of polyethylene glycol (30%)/sodium chloride (1.6M), and incubated at room temperature for 2hr. Samples were centrifuged (10,000g for 20min) and the partially purified nucleic acid pellet resuspended in 20ml of TE (10mM Tris-HCl, 1mM sodium EDTA, pH 8.0). Potassium acetate (7.5M) was added to a final concentration of 0.5M. Samples were transferred to ice for 5min then centrifuged (16,000g, 30min) at 4°C to precipitate proteins and polysaccharides. The aqueous phase was extracted with phenol/chloroform and chloroform/isoamyl alcohol [12] and DNA was precipitated by adding 0.6 volume isopropanol. After 2 hrs at room temperature, DNA was pelleted by centrifugation (16,000g for 30min) and resuspended in TE (1ml).

2.4. DNA Extraction Using Sonication (Modified from 13)

Extraction buffer (100ml) was mixed with soil (50g) on ice. The mixture was sonicated using a High Intensity Ultrasonic Processor (Vibra Cell) with a standard 13mm horn solid probe for 150 seconds. The sample was cooled in ice and the sonication repeated. SDS was added (10ml; 20%) and the sample incubated at 65°C for 1hr. The sample was transferred to centrifuge bottles (250ml) and centrifuged at 6000g for 10min. The supernatant was collected, and the soil pellet re-extracted with further extraction buffer (50ml), incubation at 65°C for 10 minutes and centrifuged as above. Extraction was then continued as per bead beating method (Figure **2**).

2.5. DNA Extraction Using Enzymatic Lysis (Modified from 11)

Extraction buffer (100ml) containing proteinase K (5mg) was mixed with soil (50g) in 250ml centrifuge tubes. The sample was incubated at 37°C for 30 minutes with shaking at 180rpm. SDS was added (10ml; 20%) and the sample incubated at 65°C for 90min. The supernatant was collected after centrifugation at 6000g for 10min at room temperature. Extraction was continued as per bead beating method.

2.6. DNA Extraction from Bacterial Cells Isolated from Soil (Modified from 4 and 14)

The bacterial fraction of soil was separated from the inorganic or humic layer by a differential centrifugation technique [14]. Bacterial cells were lysed using lysozyme and the DNA purified using ammonium acetate precipitation and ethanol precipitation [14]. DNA was resuspended in TE.

3. TEST FOR CO-EXTRACTION OF CONTAMINANTS

Co-extracted humic acids are the major contaminant when DNA is extracted from soil. These compounds absorb at 230nm whereas DNA absorbs at 260nm and protein at 280nm. To evaluate the purity of the extracted DNA, absorbance ratios at 260nm/230 nm (DNA/humic acids) and 260nm/280nm (DNA/ protein) were determined (see Tables **2** and **3**).

Table 2: Comparison of DNA Extraction Methods Using a Single Soil

Method*	Number of Samples	$A_{260/230}$	$A_{260/280}$
Bacterial cells	4	0.83 ± 0.03	1.10 ± 0.003
Chemical lysis	10	1.06 ± 0.03	1.31 ± 0.03
Sonication	4	1.20 ± 0.10	1.41 ± 0.07
Bead beating	6	1.82 ± 0.05	1.69 ± 0.02

*DNA dilute 1:100.

Table 3: Crude DNA Ratios for Different Soil Samples Extracted Using Bead Beating

Samples*	Soil Type	$A_{260/230}$	$A_{260/280}$
Western Sydney	Clay loam	1.22	1.42
Macquarie University	Clay loam	1.83	1.71
Ku-Ring-Gai Chase	Loamy sand	1.03	1.30
Balmain Power Station	Loamy sand	1.33	1.53

*DNA dilute 1:100.

4. POLYMERASE CHAIN REACTION (PCR)

DNA (1ml of 1:50 dilution) was mixed with 9ml of Genereleaser™ (Bioventures Inc., Murfreesboro, Tennessee, USA) in a 0.5ml tube and overlaid with 2 drops of sterile mineral oil. Genereleaser™ is a proprietary agent that sequesters inhibitors of PCR. Negative controls containing water only, and Genereleaser™ only, were included in each set of reactions. Reaction tubes were heated on the high setting of a 650Watt microwave oven for 7min (4550W/min) in a microwave transparent rack (Bioventures Inc.). An Erlenmeyer flask containing 100ml of water was included as a microwave sink. Tubes were incubated for at least 10 min at 80°C in an Omn-E PCR machine (Hybaid). PCR master mix (40μl) was then added to each tube. Final concentrations of reagents were as follows: 20mM $(NH_4)_2SO_4$, 75mM Tris-HCl (pH 9.0), 0.01% (w/v) Tween 20, 2mM $MgCl_2$, 0.5mM of each primer, 0.2mM of each deoxyribonucleotide triphosphate, and 1 U Red Hot DNA Polymerase (Advanced Biotechnologies, Surrey, UK). The following thermal cycle was performed: 94°C 3min (1 cycle), 94°C 1min, 55°C 1min, 72°C 2min (35 cycles), 72°C 5min (1 cycle).

5. GEL ELECTROPHORESIS

An aliquot (7μl) of each amplification reaction was analysed on 2% w/v agarose gels cast and run in TBE buffer (pH 8.3) (12). Gels were stained with ethidium bromide and photographed using transmitted U.V. light and Polaroid film (12). A 100 base pair marker (Pharmacia, LKB) was included on every gel.

6. RESULTS AND DISCUSSION

DNA extraction from soil has three requirements: extraction of high molecular weight DNA; extraction of DNA free from inhibitors for subsequent molecular biological manipulations to be performed; and representative lysis of microorganisms within the sample. In this paper, we tested a number of DNA extraction methods for their ability to fulfill these requirements.

DNA extracted using sonication was more degraded than for the other methods tested. The size of DNA extracted ranged from less than 500bp to greater than 20kb in size. Methods that shear DNA, such as sonication, generally result in DNA of 100-500bp [13]. Higher molecular weight DNA is desirable for PCR since the greater the size of the DNA, the less likely is the formation of chimeras during PCR [15]. The bead beating method used here performed better than those previously reported which usually extract DNA of less than 10kb in size [3]. The DNA extraction methods that did not use sonication all produced DNA of greater than 20kb.

Organic matter is the major source of inhibitors that may be co-extracted from soil with the microbial DNA. In particular, humic acids pose a considerable problem

and will interfere in enzymatic manipulations of DNA [5, 14, 16]. DNA polymerases have been found to be inhibited by as little as 1µl of undiluted humic-acid-like extract, regardless of the amount of DNA present [16].

The humic materials in soil have similar size and charge characteristics to DNA resulting in their co-purification [17], evident by the extractions being brown in colour. Humic contaminants also interfere in DNA quantitation since they exhibit absorbance at both 230nm and at 260nm, the later used to quantitate DNA. This characteristic can be used to determine the level of contamination of humic material by examining absorbance ratios. A high 260/230 ratio (>2) is indicative of pure DNA, while a low ratio is indicative of humic acid contamination and a high 260/280 ratio (>1.7) is indicative of pure DNA, while a low ratio is indicative of protein contamination. When the DNA extraction methods were compared (Table **2**), the bead beating method consistently extracted DNA with higher 260/230 and 260/280 ratios. This indicated that the DNA was contaminated with fewer humic acid-like compounds. Although the extracts were still brown in colour, dilution of the DNA to 1:50 from all methods was suitable to produce a PCR product. Heavy metal ions, such as are present in the Balmain soil (Table **1**), also contribute to inhibitory effects [18]. Here we have demonstrated that a PCR product from soil DNA contaminated with humic acids and heavy metals can be obtained without the use of expensive purification products.

To determine the diversity of microorganisms from which DNA had been extracted, different primer sets were tested [4], including both multi- and single-copy genes. The multi-copy targets included the prokaryotic small subunit rRNA [19], prokaryotic rRNA intergenic spacer region [20], the eukaryotic rRNA internal transcribed spacer (ITS) region [21], the ITS region for lichen fungi [22], and the HSP70 family of proteins [23] while the low abundance targets included fungal *β*-tubulin [24], and *nif*H genes [25]. With dilution of DNA from each extraction technique, successful PCR amplification was achieved with all primers tested (see Figure **1**).

Due to ease of the method, the reduced co-extraction of inhibitors (Tables **2** and **3**) and the greater confidence that bead beating would lyse all microbial cells in the soil, this was the method of choice and concentrated on for further analysis (see [1]). Bead beating has been found to have a lysis efficiency of greater than 90% [3]. The PCR results reported here

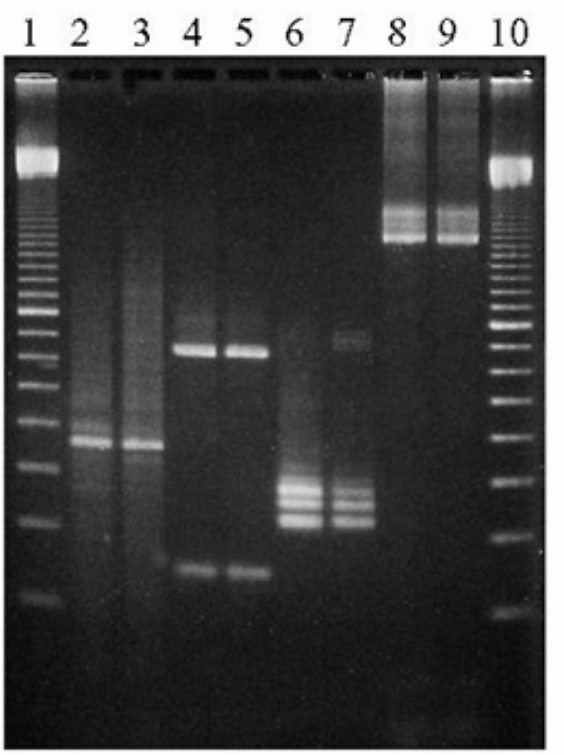

Figure 1: Example of PCR amplification products using various DNA targets with soil extracted by enzymatic lysis or bead beating. Lane 1: 100bp marker; lane 2: enzymatic lysis DNA with 16S rRNA primers [19]; lane 3: bead beating DNA with 16S rRNA primers [19].

provide further evidence to support this with products from both bacterial and fungal elements of the soil microbiota being obtained. The bead beating direct lysis method described here extracts between 1.5 and 2.35mg/ml of DNA from 100g of soil or 15-23.5 µg DNA/g soil. Extraction methods using small soil samples ranging from 5g to 100mg of soil have extracted 9-25µg DNA/g soil [6], 12µg/g [18], 1-100µg/g [26], and 2.5-26.9µg/g [11]. The method described here is therefore at least as efficient as the above methods.

The focus of DNA extraction methods has moved to rapid performance of molecular techniques, avoiding extensive purification steps [7, 27]. Using the bead beating DNA extraction method described here, crude microbial DNA could be extracted from a variety of soil types and dilution of this DNA was sufficient for successful PCR from both high- and low-copy number genes.

ACKNOWLEDGMENTS

Thanks are due to Semnan University Department desert science for supplying soil from Semnan Agriculture Station and Semnan University.

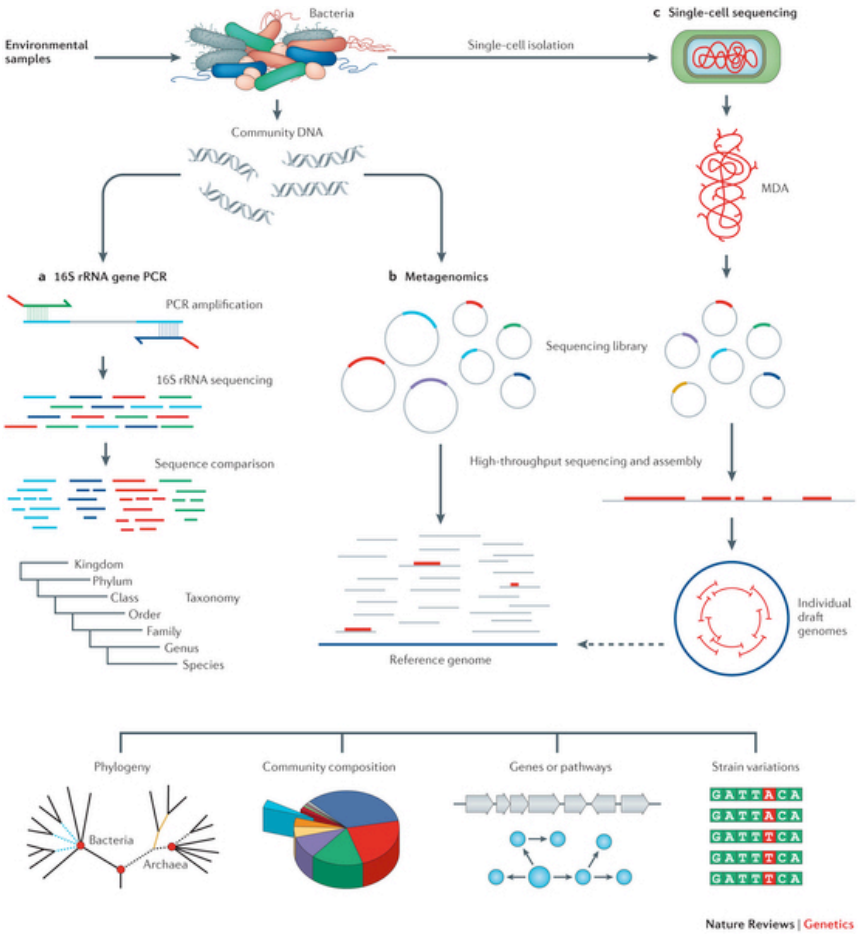

Figure 2: a: PCR amplification of the 16S ribosomal RNA gene can be carried out from most novel bacteria using primers that anneal to sequences which are highly conserved across bacterial species. Variable regions of the 16S rRNA gene can then be used to derive a phylogenetic tree. **b:** Metagenomics is based on sequencing of total DNA extracted from the environment. Gene content and frequencies are obtained for the entire ecosystem; however, assembly of the sequencing reads from the sequencing library inserts (which are indicated by coloured sequences) into the genomes of individual species is complicated by the large number of organisms that contribute to the DNA in the sample. **c:** Single-cell sequencing typically does not recover the entire genome; however, the reads obtained in the sequencing library are all genetically linked, which facilitates genome assembly. These genomes represent the integrated genetics and biochemistry of individual microbial species. Single-cell genomes can serve as a reference genome to aid the assembly of sequencing data for uncultivated species that are closely related. DNA or cDNA sequencing reads obtained through shotgun metagenomic or metatranscriptomic approaches can be mapped to the reference genomes (dashed arrow). Single-cell reference genomes can also provide phylogenetic placement for a substantial portion of the metagenomic reads that have not been previously assigned a taxonomy. 16S rRNA gene PCR is a rapid and inexpensive method to assign phylogeny and community composition. Metagenomics also provides community composition and gene content. Single-cell sequencing provides assembled contigs to determine genes and pathways within an individual cell.

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Received on 01-11-2015 Accepted on 10-12-2015 Published on 30-12-2015

DOI: http://dx.doi.org/10.15377/2409-9813.2015.02.02.2