# A comparative study of extraction and purification methods for environmental DNA from soil and sludge samples

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## Abstract

An important prerequisite for a successful metagenome library construction is an efficient extraction procedure for DNA out of environmental samples. In this study we compared three indirect and four direct extraction methods, including a commercial kit, in terms of DNA yield, purity and time requirement. A special focus was set on methods which are appropriate for the extraction of environmental DNA (eDNA) from very limited sample sizes (0.1 g) to enable a highly parallel approach. Direct extraction procedures yielded on average 100-fold higher DNA amounts than indirect ones. A drawback of direct extraction was the small fragment size of appr. 12 kb. The quality of the extracted DNA was evaluated by the ability of different restriction enzymes to digest the environmental DNA. Only the commercial kit and a direct extraction method using freeze-thaw cell lysis in combination with an in gel patch electrophoresis with hydroxyapatite to remove humic acid substances yielded DNA, which was completely digested by all restriction enzymes. Moreover, only DNA extracted by these two procedures could be used as template for the amplification of fragments of several 16S rDNA, 18S rDNA groups under standard PCR conditions.

## Introduction

Cultured microbes have revealed tremendous chemical resources and utility, however the vast majority of as yet unknown microorganisms from environmental samples could well be a far larger source of new molecular structures. The current estimates indicate that less than 1% of the total microbial community observed under the microscope can be cultivated with known cultivation techniques (1-9). There is likely to be further diversity within species (10-12), which current phylogenetic analysis cannot resolve. The methodological protocols for understanding aspects of these resources have been dramatically developed in recent years. Until now, numerous researchers have improved methods for extracting pure environmental DNA (eDNA). Especially, the problem of co-extracting humic substances which severely inhibit PCR amplification reactions and cloning procedures were addressed in several publications (13-28). These different methods for the extraction of eDNA from environmental microbes vary in the degree of shearing, purity and yield of the isolated eDNA. While many of these protocols have been employed for years, only recent studies have begun to compare their efficiency and reliability (15,16). There are basically two different approaches to extract eDNA. Either the cells are separated from the environmental samples prior the cell lysis (indirect cell extraction) or the microbes are lysed within the environmental samples (direct cell

extraction).

In this study, three indirect and four direct cell extraction methods, including one commercially available procedure, were compared in regard of the DNA yield and purity after extraction and purification from environmental samples. The quality of the obtained eDNA was verified by several PCR amplification reactions and restriction enzyme digestions.

## Material and Methods

### Sampling

Soil was collected in Stuttgart at the Institute of Technical Biochemistry (University of Stuttgart, Germany). Sediment was collected from a settling pond in the lake Bärensee in Stuttgart (Germany). Activated sludge was collected from a sewage plant at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management of the University of Suttgart (Germany). All methods were carried out with 0.1 g sample, stored at –20 °C before use. All samples were expressed as wet weights.

### Indirect Extraction Methods

**Method 1 (Quaiser *et al* 2002):** Environmental samples (0.1 g) were collected and suspended in 1 ml buffer (20 mM Tris-HCl, 10 mM EDTA, 10 mM ε-aminocaproic acid, pH 8.0) and incubated at 4°C for 10 h with shaking. The particles were collected by low speed centrifugation (3000 rpm) for 5 min. The microbial cell fraction was centrifuged and embedded into an agarose plug. The plug was incubated at 37°C for 1 h in extraction buffer (100 mM EDTA, 10 mM Tris-HCl, 50 mM NaCl, 0.2% (w/v) deoxycholate, 1% (w/v) N-lauroyl sarcosine, 1 mg/ml lysozyme, pH 8.0), then transferred into a buffer, containing 2 mg/ml proteinase K, 1% (w/v) lauroyl sarcosine, 500 mM EDTA and incubated overnight at 50°C with gently shaking. Agarose plugs were placed in a 0.5% agarose gel which contained 2% polyvinylpyrrolidone (PVP) in the first half and no PVP in the other part. Gel electrophoresis was performed overnight at 4°C, 5 V/cm. The eDNA was extracted from gel with the QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany), the resulting DNA was dialysed (20mM Tris-HCl, pH 7.8, 10 mM NaCl, 0.2 M EDTA) and concentrated in an evaporator. For the analysis of the fragment size 10 g environmental sample was suspended in 10ml buffer (Tris-HCl, 10 mM EDTA, 10 mM ε-aminocaproic acid, pH 8.0). The following procedures for eDNA extraction was the same as described above for method 1.

**Method 2 (Holben *et al* 1994):** Environmental samples (0.1 g) were collected and suspended in 1 ml buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) cetyltrimethylammonium bromide (CTAB), pH 8.0) and homogenized in a blender (Ultra-Turrax T25, Germany) for three 1 min intervals. The particles were collected by low speed centrifugation (3000 rpm) for 10 min. The supernatant was centrifuged at high speed (14000 rpm) for 20 min. After cells collecting, they were washed in 1 ml Chrombach buffer (300 mM Tris-HCl, 1 mM EDTA, pH 8.0), the pellet was resuspended in 100 µl lysis buffer, lysozyme (50 mg/ml) and proteinase K (10 mg/ml) solution, and incubated at 37°C for 30 min. Lysis was carried out chemically by the addition of 100 µL 20 % (w/v) SDS solution and incubated for 1 h at 65°C. After phenol : chloroform : isoamylalcohol (25:24:1) extraction, DNA was precipitated with isopropanol and then the pellet was subsequently precipitated with 70% ethanol. Crude DNA pellets were dissolved in 100 µl TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Crude DNA extracts were purified by gel electrophoresis using 1% (w/v) agarose gels. eDNA was extracted from gel with the QIAEX II gel extraction kit (QIAGEN, Hilden, Germany). For the analysis of the fragment size 10 g environmental sample was suspended in 10ml buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v)

cetyltrimethylammonium bromide (CTAB), pH 8.0. The following procedures for eDNA extraction was the same as described above for method 2.

**Method 3 (Jacobsen *et al* 1992):** Environmental samples (0.1 g) were collected and 0.1 g Chelex 100 (Bio-Rad Laboratories, Richmond, USA) was added to 1 ml of 0.1 % (w/v) Na-deoxycholate and 2.5 % (w/v) polyethylene glycol (PEG) 6000 solution. After shaking at 4°C for 1 h, environmental samples and Chelex 100 were removed by low speed centrifugation (3000 rpm). The microbial cell fraction was collected by centrifugation and washed in 500 µl TE buffer (33 mM Tris-HCl, 1 mM EDTA, pH 8.0). Lysis was performed chemically with 100 µl of 20 % (w/v) SDS and 500 µl of 5 M NaCl with lysozyme (10mg/ml) and incubated for 1 h at 65 °C. The supernatant was collected by centrifugation and suspended in 500 µl TE buffer (pH 8.0). DNA was purified by ion exchange chromatography with a Q-Sepharose column (25ml volume, Pharmacia Biotech, Erlangen, Germany) with linear gradient (Eluent A : 10 mM Tris-HCl, 1mM EDTA, pH 8.0, Eluent B : 10 mM Tris-HCl, 1mM EDTA, 1 M NaCl, pH 8.0). For the analysis of the fragment size 10 g environmental sample and 10g Chelex 100 was added to 10ml of a 0.1 % (w/v) Na-deoxycholate solution containing 2.5 % (w/v) PEG 6000. The following procedures for eDNA extraction was the same as

described above for method 3.

## Direct Extraction Methods

**Method 4 (Orsini *et al* 2001):** Environmental samples (0.1 g) were collected and suspended in 1 ml of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 0.1 % (w/v) SDS, 0.1 % (w/v) PVP, pH 8.0). Samples were centrifuged at 8000 rpm for 1 min and resuspended in 500 µl of a lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 3 % (w/v) SDS, 1 % (w/v) PVP, pH 8.0). Tubes were heated in a microwave oven at 600-700 W for 1 min. A 500 µl volume of pre-warmed (65 °C) extraction solution (10 mM Tris-HCl, 1 mM EDTA, 300 mM sodium acetate, 1 % (w/v) PVP) was added to the sample. Phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by inversion. DNA precipitation was performed with isopropanol and the DNA pellet was washed with 70% ethanol and resuspended in 100 µl of TE buffer (pH 8.0).

**Method 5 (Yeates *et al* 1997):** Environmental samples (0.1 g) were collected and suspended in 1 ml extraction buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 1.5 M NaCl, pH 8.0). Glass beads (0.1 g, 0.5 mm, Biospec product Inc.) were added and the sample blended in a Bead-Beater (Retsch, Germany) for 20 min. A 100 µl of 20 % (w/v)

SDS was added and incubated at 65 °C for 1 h and centrifuged at 8000 rpm for 10 min. The supernatants were transferred to tubes containing a 500 µl of PEG solution (30 % (w/v) PEG, 1.5 M NaCl) and incubated at room temperature for 2 h. Samples were centrifuged (14000 rpm) for 20 min. The aqueous phase was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and DNA was precipitated by adding 0.6 volume isopropanol. DNA was collected by centrifugation (14000 rpm) for 30 min. Conventional electrophoresis was used to remove humic substances. DNA was purified using a QIAEX ll Gel Extraction Kit (QIAGEN, Hilden, Germany) and resuspended in TE buffer (pH 8.0).

**Method 6 (Roh *et al* 2005):** Environmental samples (0.1 g) were collected and suspended in 1 ml of TENC buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% (w/v) CTAB, 1µl of 50 µg/µl Proteinase K, pH 8.0). The samples were frozen in liquid nitrogen for 5 min and subsequently thawed at 65 °C for 10 min. Then, 100 µl of 20% (w/v) SDS was added and the sample was incubated at 65 °C for 10 min. After centrifugation at 14000 rpm for 10 min, the supernatant was transferred into a sterile tube. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the combined supernatant and mixed by inversion. The supernatant was collected and 1 ml

isopropanol and 70% ethanol was added before centrifugation at 14000 rpm for 5 min. Crude DNA was resuspended in 50 µl of TE buffer (pH 8.0). Crude DNA was loaded onto 0.5 % agarose gel. One part of the agarose gel contained hydroxyapatite to remove humic substances (29). A volume of 40 mL melted 0.5 % low melting point (LMP) agarose was gently mixed with 2 g of hydroxyapatite resin (Fluka, Buchs, Ch). The mixture was poured into sterile rectangular molds and rotated with ROTAMIX RM1 (ELMI) to ensure a uniform distribution of the resin in the agarose gel prior to solidification. Then the gel patch was extracted from the mold. After fixing the gel patch in the electrophoresis rack, melted 0.5 % LMP agarose was poured in the rack and then electrophoresis was carried out at 120V for 20min. Subsequent DNA purification was carried out with the QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany).

**Method 7 (*SoilMaster*™ DNA Extraction Kit (EPICENTRE, Madison, Wisconsin, http://www.epibio.com):** The eDNA of environmental samples was extracted by the *SoilMaster*™ DNA Extraction Kit according to the manufacturer's recommendations. Environmental samples (0.1 g) were collected and suspended in 250 µl of DNA extraction buffer. Two microliter of Proteinase K and 50 µl lysis buffer were added. The sample was incubated at 65 °C for 10 min. After centrifugation at 3000 rpm for 2 min,

the supernatant was transferred to a sterile tube. For protein precipitation 60 µl of protein precipitation reagent was added and mixed thoroughly by inverting. After centrifugation, the supernatant was placed directly on the spin column with the inhibitor removal resin. After repeating wash and spin, the precipitated eDNA was resuspended in 300 µl TE buffer.

## Restriction endonuclease treatment and polymerase chain reaction

Restriction enzyme digestions were carried out to examine the digestibility of eDNA obtained by four different direct extraction methods. The digestions (BamHI, EcoRI, HindIII, SmaI, XhoI; Fermentas) were done under conditions provided by the manufacturer. After incubation for 30 min, the DNA fragments were resolved in a 1 % agarose gel. Purified eDNA extracted from environmental samples was used as template in the polymerase chain reaction (PCR). PCR amplification was conducted using *Taq* DNA polymerase (Eppendorf, Germany) under conditions provided by the manufacturer. PCR was conducted with a Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) as follows: 2 min of denaturation at 94 °C, followed by 35 amplification cycles (1 min at 94 °C, 1 min at the optimized annealing temperature (Table 4), 1 min at 72 °C (extension), with a final 10 min 72°C extension step after cycling was complete.

### Determination of purity and yield of eDNA

To evaluate the purity of the extracted DNA, absorbance ratios at 260nm/230nm and 260nm/280nm were determined (27). A high $A_{260}$ to $A_{230}$ ratio of > 2 indicates pure DNA. A low ratio indicates contamination with humic substances. A $A_{260}$ to $A_{280}$ ratio of less than 1.7 indicates protein contamination. The quantification of the extracted eDNA was also carried out using a spectrophotometric method (27).

### DNA sequencing

The DNA sequencing reaction was carried out on both strands of double-stranded templates using the BigDye Terminator Cycle Sequencing Kit RR-100 (Applied Biosystems, Weiterstadt, Germany). Sequencing was performed with an ABI Prism$^{TM}$ 377 DNA Sequencer (Perkin Elmer, Shelton, USA).

## Results

Three indirect and four direct DNA extraction methods from environmental samples were compared in terms of DNA yield, purity and time requirement. For the direct extraction methods, the cell lysis was performed in the presence of the sample matrix,

whereas the indirect approach involves the extraction of cells prior the cell lysis. The examined methods mainly differ in the type of cell lysis and DNA purification (Table 1). The direct extraction methods include microwave thermal shock (method 4), mechanical disruption by bead beating (method 5), freeze-thawing cycles combined with in gel patch electrophoresis (method 6). In the commercial kit a hot detergent lysis process was combined with spin column purification (method 7). In the indirect extraction methods cells were lysed enzymatically with lysozyme. In all cases a sample volume of 0.1 g was used in order to compare the results with that of the tested commercial kit. The DNA yields of the three examined indirect extraction methods lay between 0.02 and 0.06 µg/g soil [wet wt] (Table 2), with the highest DNA concentration obtained with method 2 from Holben *et al*. Direct extraction methods yielded about 100-fold higher DNA concentrations in the range between 1.1 and 11.4 µg/g soil. The highest DNA concentrations of 9.7 µg/g on average from environmental samples were obtained with method 6, whereas the commercial kit yielded the lowest DNA concentrations (1.5 µg/g) of the four examined direct extraction methods. There were no significant differences observed between the DNA yields received from the different types of environmental samples, the soil, sediment or activated sludge.

The cost of the extraction efficiency of direct extraction methods is the resulting eDNA

fragments of only around 12 kb in size due to shearing processes (Fig. 1). Starting with 0.1 g of environmental sample, no bands were visible on the agarose gel in the case of indirect extraction methods. However, when the indirect extraction procedures were applied to 10 g soil sample, very large DNA fragments could be obtained (Fig. 2). The DNA obtained by indirect extraction methods 1-3 had very little size distribution. There was a clear band of appr. 40 kb in the case of method 1 and 2 and even larger DNA fragments of more than 50 kb by method 3 with nearly no smear of smaller DNA fragments.

The $A_{260}/A_{280}$ ratio corresponding to direct extraction methods was in all cases at least 1.7, indicating a sufficient removal of protein contaminations. In the case of indirect extraction methods, only the method 3 applied to soil and sediment and method 1 applied to activated sludge produced a value of 1.7. Concerning the removal of humic acid contaminations, indicated by a $A_{260}/A_{230}$ ratio of more than 2, only the method 6 gave satisfactory results.

As can be seen in Table 1, indirect extraction methods were more time-intensive than direct extraction methods because of the additional matrix removal step. Methods 1, 2 and 3 required 48 h, 4 h and 7h, respectively. Whereas methods 4 and 7 were the fastest procedures with 30 min and 1 h. Methods 5 and 6 required 5.5 h and 2.5 h, respectively.

The purity of the extracted eDNAs was investigated by the ability of several restriction enzymes to digest it. Table 3 shows the results of the restriction enzyme treatment of eDNA obtained by direct extraction methods. None of the crude extracts could be digested by the five restriction enzymes BamHI, EcoRI, HindIII, SmaI, and XhoI. Furthermore, although a reduction in the dark brownish color of the crude eDNA was observed after microwave thermal shock (method 4) and mechanical cell lysis in combination with conventional gel electrophoresis (method 5), eDNA extracted by the method 4 still could not be digested by BamHI, EcoRI or HindIII. SmaI and XhoI caused a partial digestion of the extracted DNA. eDNA extracted by the method 5 could not be digested by Bam HI or Eco RI and only partially digested by Hind III, SmaI and XhoI. However, when purified by method 6 and method 7, extracted eDNA was completely digested by all five restriction enzymes (Table 3). Restriction digestion could not be performed with eDNA purified by indirect extraction (method 1, 2 and 3) due to low DNA concentrations received by 0.1 g sample.

The quality of the eDNA purified by the four direct extraction methods was also investigated by the ability to amplify a region of the rRNA of several phylogenetic groups representing different components of the microbial communities. These groups include members with a broad range of microbial cell sizes and growth habits. Six

different sets of primers were used (Table 4). 16S rDNA and 18S rDNA amplification analysis by method 6 and 7 resulted in successful PCR amplifications of gene fractions of the expected size (Fig. 3). All targeted microbial groups were detected under standard PCR conditions using eDNA as template. The PCR result of Gram-positive bacteria with high G+C content proved the lysis of bacteria. Detection of the prokaryotic small subunit rRNA, the internal transcribed spacer (ITS) region for lichen fungi, which represent the fungal part of the symbioses of a fungus and a cyanobacteria in lichens, and the eukaryotic rRNA internal transcribed spacer (ITS) region was very clearly at the three types of environmental samples extracted by method 7. The corresponding results obtained by method 6 were already described in a former publication (29). Whereas, using the eDNA extracted by method 4 and 5 as template for the PCR no amplification product was observed. Negative controls without eDNA as template also resulted in no PCR product (data not shown).

## Discussion

In this study, the applicability of different soil extraction and DNA purification methods were examined to obtain suitable eDNA for molecular biology procedures from very small sample sizes. The sample size was limited to 0.1 g because this amount enables a

highly parallel sample processing. The results of this work showed that this small sample size is insufficient for indirect extraction methods. This correlates with former descriptions of indirect extraction procedures where usually 10 g - 100 g soil was used to get enough DNA for further cloning steps (30-32). Garbor *et al*. reported 10 to 100-fold lower DNA yields obtained by indirect extraction methods compared to direct DNA extraction (15). Also, Tien *et al*. obtained on average a 10 times higher DNA amount with direct lysis methods compared to indirect extraction (20). The overall highest DNA yield in this study was obtained by the method 6, which was recently developed by our group (29). Freeze-thaw cell lysis in combination with gel electrophoresis with a hydroxyapatite patch inside the agarose gel resulted in higher DNA amounts than mechanical cell lysis by bead-beating combined with conventional gel electrophoresis. Miller *et al*. compared the efficiency of bead-beating cell lysis with that of freeze-thaw lysis and came to the result that bead-beating is superior to the freeze-thaw technique (33). So, the increased DNA amount obtained by the method 6 compared to the method 5 might be related to the different purification procedures. Moreover, in terms of purity and humic acid removal, method 6 gave the best results, followed by the examined commercial kit. The hydroxyapatite resin present in a part of the agarose gel causes an effective binding of the humic substances. Alternatively, CsCl-ethidium bromide

equilibrium density centrifugation or purification procedures using a chromatographic column might be used to obtain pure enough DNA, but these methods are time-consuming and the former method requires an ultracentrifuge (25, 34).

A disadvantage of direct extraction methods is the rather small DNA fragment size. Similar to the results of this work Lloyd-Jones *et al*. obtained DNA fragments of around 12 kb by direct extraction methods using chemical lysis and bead beating cell lysis (16). However, there are several examples of metagenomic libraries derived from eDNA from direct extraction procedures, which resulted in the discovery and heterologous expression of new enzymes (32, 35-38). The fragment sizes obtained by indirect extraction methods are substantially higher (15). Fragment sizes extracted from soil can reach up to 400 kb (30).

The big advantage of direct extraction methods is that they are much faster than indirect extraction methods. The SoilMaster commercial kit provided eDNA, which was sufficiently pure to be used as template for PCR after 1 hour, whereas the purity of the extracted DNA could be further enhanced by the in gel patch electrophoresis (method 6). This method requires about 2.5 h, which is still suitable for high-throughput screening.

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## Tables

**Table 1:** Comparison of examined indirect and direct DNA extraction methods.

**Table 2:** Amount and Purity of eDNA extracted from environmental samples.

**Table 3:** Restriction enzyme digestion of eDNA purified from environmental samples.

**Table 4:** Primer Pairs used in PCR to detect phylogenetic groups of native microorganisms in environmental samples.

## Figure Legends

**Figure 1:**

Comparison of different methods for extraction of eDNA from environmental samples. M, DNA marker; eDNA extracted by method 1 from soil (1), sediment (2) and activated sludge (3); eDNA extracted by method 2 from soil (4), sediment (5) and activated sludge (6); eDNA extracted by method 3 from soil (7), sediment (8) and activated sludge (9); eDNA extracted by method 4 from soil (10), sediment (11) and activated sludge (12); eDNA extracted by method 5 from soil (13), sediment (14) and activated sludge (15); eDNA extracted by method 6 from soil (16), sediment (17) and activated sludge (18); eDNA extracted by method 7 from soil (19), sediment (20) and activated sludge (21).

**Figure 2:**

Environmental DNA (eDNA) extracted by indirect methods of 10g soil sample by method 1 (a), method 2 (b) and method 3 (c); M: Lambda mix marker.

**Figure 3:**

PCR amplification analysis of eDNA extracted from environmental samples by method

6: (a), by method 7: (b).

(a): M, DNA marker; bacillus species and relatives region from soil (lane 1), sediment (lane 4) and activated sludge (lane 7); streptomyces species and related taxa region from soil (lane 2), sediment (lane 5) activated sludge (lane 8); high G+C gram-positive bacteria region from soil (lane 3), sediment (lane 6) activated sludge (lane 9).

(b): M, DNA marker; prokaryotic small subunit rRNA region of soil (lane 1), sediment (lane 7) and activated sludge (lane 13); bacillus species and relatives region of soil (lane 2), sediment (lane 8) and activated sludge (lane 14); streptomyces species and related taxa region of soil (lane 3), sediment (lane 9) and activated sludge (lane 15); high G+C gram-positive bacteria region of soil (lane 4), sediment (lane 10) and activated sludge (lane 16); internal transcribed spacer (ITS) region for lichen fungi of soil (lane 5), sediment (lane 11) and activated sludge (lane 17); eukaryotic rRNA internal transcribed spacer (ITS) region of soil (lane 6), sediment (lane 12) and activated sludge (lane 18).

Table 1: Comparison of examined indirect and direct DNA extraction methods.

| | **Indirect Extraction Method** | | | **Direct Extraction Method** | | | |
|---|---|---|---|---|---|---|---|
| | Quaiser *et al* (2002) Method 1 | Holben *et al* (1994) Method 2 | Jacobsen *et al* (1992) Method 3 | Orsini *et al* (2001) Method 4 | Yeates *et al* (1997) Method 5 | Roh *et al* (2005) Method 6 | Commercial Kit Method 7 |
| Soil removal | Gently shaking in buffer (4°C, 10 h); Low speed centrifugation | Homogenization in buffer (SDS, CTAB) Low speed centrifugation | Shaking in buffer (4°C 1 h) + cation-exchange resin Low speed centrifugation | - | - | - | - |
| Cell lysis | Agarose Plug, enzymatic lysis with lysozyme and proteinase K | Lysozyme & proteinase K + Chemical lysis (SDS, 65°C, 1h) | Lysozyme & Chemical lysis (SDS + NaCl) at 65°C, 1h | Microwave thermal shock | Bead-Beating | Freezing & thawing + proteinase K | Hot detergent lysis + proteinase K |
| Protein removal | - | P:C:I (25:24:1) | - | P:C:I (25:24:1) | P:C:I (25:24:1) | P:C:I (25:24:1) | Precipitation reagent |
| DNA purification | Two phase electrophoresis (PVP) + gel extraction | gel electrophoresis + gel extraction | Ion exchange chromatography (Q-sepharose) | - | Conventional gel electrophoresis + gel extraction | In gel patch electrophoresis (HA) + gel extraction | Spin column with inhibitor removal resin |
| Spent time | 48 h | 4 h | 7 h | 30 min | 5.5 h | 2.5 h | 1 h |

PVP: polyvinyl pyrrolidone; HA: hydroxyapatite; SDS: sodium dodecyl sulphate; CTAB: cetyltrimethylammonium bromide; PEG: polyethylene glycol; P:C:I: phenol:chloroform:isoamyl alcohol

Table 2: Amount and purity of eDNA extracted from environmental samples.

| Type of purification | | Amount DNA µg/g [wet wt][a] | | | Purity | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $A_{260}/A_{280}$ | | | $A_{260}/A_{230}$ | | |
| | | soil | sediment | activated sludge | soil | sediment | activated sludge | soil | sediment | activated sludge |
| Indirect | Method 1 | $3.0\times10^{-2}$ | $3.0\times10^{-2}$ | $1.5\times10^{-2}$ | 1.48 ± 0.03 | 1.51 ± 0.02 | 1.65 ± 0.02 | 1.73 ± 0.05 | 1.75 ± 0.04 | 1.71 ± 0.03 |
| | Method 2 | $6.0\times10^{-2}$ | $4.2\times10^{-2}$ | $2.4\times10^{-2}$ | 1.49 ± 0.03 | 1.59 ± 0.03 | 1.51 ± 0.03 | 1.62 ± 0.03 | 1.63 ± 0.04 | 1.75 ± 0.02 |
| | Method 3 | $4.5\times10^{-2}$ | $3.5\times10^{-2}$ | $1.7\times10^{-2}$ | 1.65 ± 0.02 | 1.67 ± 0.03 | 1.59 ± 0.02 | 1.67 ± 0.02 | 1.73 ± 0.05 | 1.74 ± 0.03 |
| Direct | Method 4 | 4.5 ± 1.1 | 5.1 ± 0.8 | 4.5 ± 0.7 | 1.68 ± 0.05 | 1.71± 0.05 | 1.77 ± 0.05 | 1.61 ± 0.07 | 1.68 ± 0.07 | 1.73 ± 0.05 |
| | Method 5 | 6.5 ± 0.8 | 6.7 ± 0.7 | 5.9 ± 0.8 | 1.72 ± 0.05 | 1.71 ± 0.07 | 1.81 ± 0.04 | 1.63 ± 0.03 | 1.71 ± 0.03 | 1.76 ± 0.04 |
| | Method 6 | 8.3 ± 1.2 | 9.5 ± 0.9 | 11.4 ± 0.6 | 1.89 ± 0.03 | 1.85 ± 0.02 | 1.89 ± 0.03 | 1.98 ± 0.03 | 2.03 ± 0.02 | 1.97 ± 0.03 |
| | Method 7 | 1.9 ± 0.3 | 1.5 ± 1.1 | 1.1 ± 0.5 | 1.75 ± 0.02 | 1.76 ± 0.02 | 1.85 ± 0.03 | 1.89 ± 0.03 | 1.87 ± 0.03 | 1.82 ± 0.02 |

[a] Values are means of four independently purified samples with standard errors.

The ratios were calculated from spectrophotometric measurements.

Table 3: Restriction enzyme digestion of eDNA purified from environmental samples.

| Type of purification | | Digested by | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BamHI | | | EcoRI | | | HindIII | | | SmaI | | | XhoI | | |
| | | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C |
| Direct cell extraction | Method 4 | - | - | - | - | - | - | - | - | - | ± | ± | ± | ± | ± | ± |
| | Method 5 | - | - | - | - | - | - | ± | ± | ± | ± | ± | ± | ± | ± | ± |
| | Method 6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Method 7 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

A: soil, B: sediment, C: activated sludge

+: complete digestion, ±: partially digestion, -: no digestion

Table 4: Primer pairs used in PCR to detect phylogenetic groups of native microorganisms in environmental samples.

| Organism group | Primer pair region[ref] | DNA sequence | Product (bp) | Optimized Annealing Temp(°C) |
|---|---|---|---|---|
| Bacteria | Prokaryotic small subunit rRNA (39) | 5’AGAGTTTGATCCTGGCTCAG<br>5’AGAAAGGAGGTGATCCAGCC | 1536 | 50 |
| | Bacillus species and relatives (40) | 5’AGGGTCATTGGAAACTGGG<br>5’CGTGTTGTAGCCCAGGTCATA | 600 | 55 |
| | High G+C gram-positive bacteria (40) | 5’GAGTTTGATCCTGGCTCAG<br>5’GCCATTGTAGCACGTGTGCA | 542 | 63 |
| | Streptomyces species and related taxa (41) | 5’GGCCTTCGGGTTGTAAACC<br>5’CTTTGAGTTTTAGCCTTGCGGC | 1243 | 60 |
| Fungi | Internal transcribed spacer (ITS) for lichen (42) | 5’GCGGAAGGATCATTACTGA<br>5’GGGTATCCCTACCTGATCCG | 565 | 56 |
| Eukaryote | rRNA internal transcribed spacer (ITS) (43) | 5’TCCGTAGGTGAACCTGCGG<br>5’TCCTCCGCTTATTGATATGC | 584 | 58 |

**Figure 1**

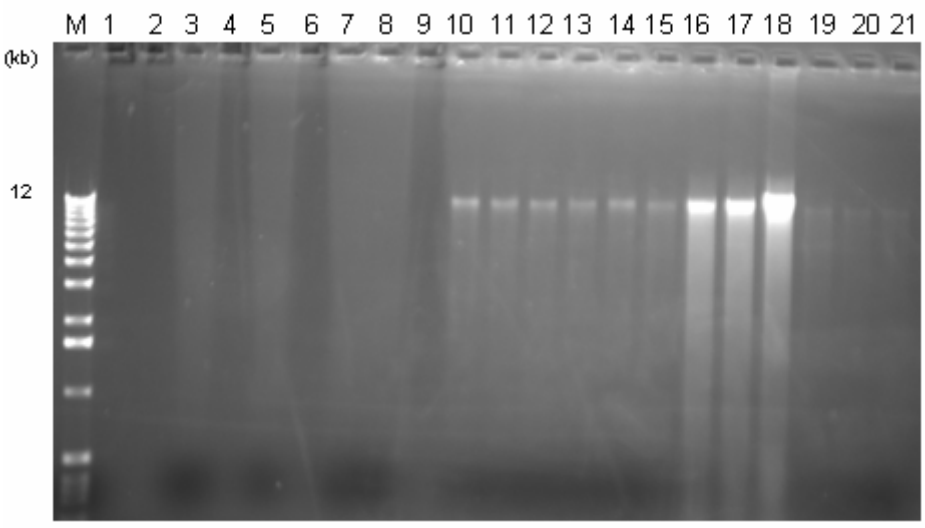


**Figure 2**

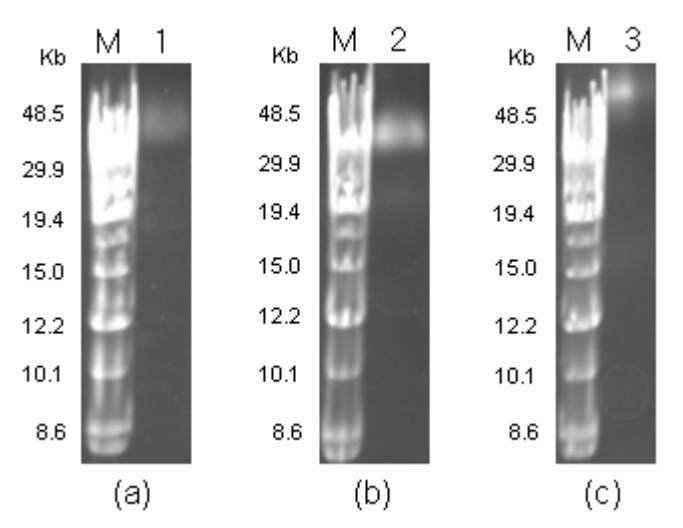
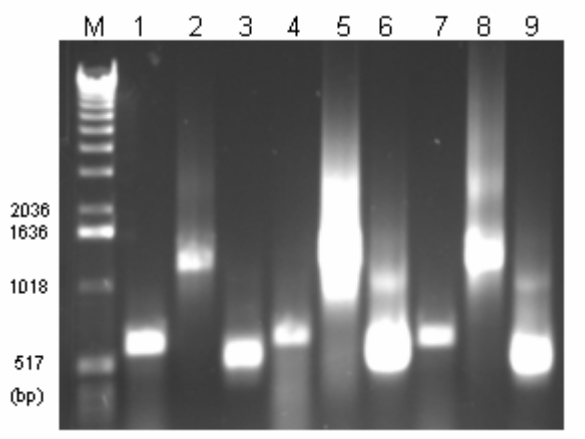
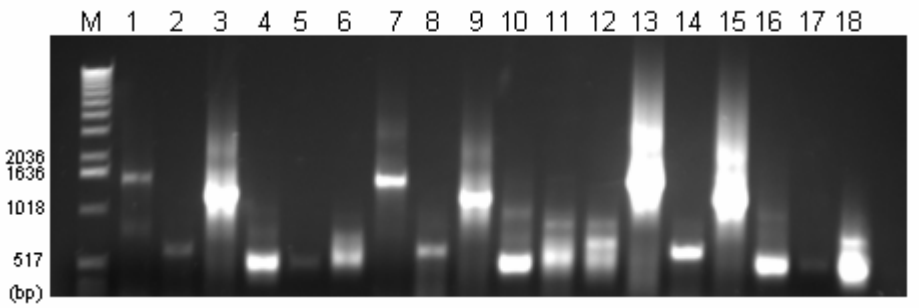

Kb
M
1
48.5
29.9
19.4
15.0
12.2
10.1
8.6
(a)
Kb
M
2
48.5
29.9
19.4
15.0
12.2
10.1
8.6
(b)
Kb
M
3
48.5
29.9
19.4
15.0
12.2
10.1
8.6
(c)

**Figure 3:**


M 1 2 3 4 5 6 7 8 9
2036
1636
1018
517
(bp)

(a)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2036
1636
1018
517
(bp)

(b)