# Testing The UltraClean™ Soil DNA Purification Kit on A Diverse Range of Soils by PCR Amplification of 16S rDNA

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## Abstract

Extracting PCR-quality DNA directly from soil has become an essential step in the study of microbial communities and for the analysis of introduced organisms in environmental samples. A simple and reliable small-scale procedure using less than 1 gram of sample, developed and sold by Mo Bio Laboratories, Inc. was used in this study to extract and purify DNA from many different soil types. The method was tested on soils representing a broad range of organic carbon and nitrogen content and varying sand/silt/clay compositions. Replicate soil samples were extracted and the resulting DNAs quantified by PicoGreen analysis. The quality of the DNA samples was determined by PCR amplification using consensus primers specific for a 530 bp region of the bacterial 16S ribosomal DNA. The extraction procedure used a combination of hot detergent treatment and a vortex-based, mechanical lysis to produce PCR quality DNA with minimal shearing from most soils in approximately 30 minutes. The DNAs recovered were directly amplified by PCR in most cases. In soils with high organic carbon content, the addition of an optional reagent during the extraction process helped alleviate the carry-over of PCR inhibitors such as humic acids

# Introduction

The use of DNA-based molecular methods has become a standard practice in the study of environmental microbes. DNA detection methods are powerful tools for detecting non-culturable organisms and for monitoring introduced organisms in the environment. Such methods rely on the Polymerase Chain Reaction (PCR) to amplify specific genes of interest directly from community DNA. However, DNA recovered from soils is problematic. Many soils contain organic compounds known to inhibit restriction endonucleases (7) and *Taq* polymerase, the key enzyme of the polymerase chain reaction (PCR) (14). Humic substances form a major component of soil organic matter. Their chemical composition is poorly understood and they are generally classified into three fractions based on water solubility. These include humin, humic acid and fulvic acid. Humin is the fraction not soluble in water at any pH. Humic acid is insoluble under acidic conditions ($pH < 2$) but becomes soluble at higher pH. Fulvic acid is soluble at all pH conditions (1). Humic and fulvic acid, are commonly found in aquatic, soil and sediment environments (12). These compounds readily co-extract with DNA and are difficult to remove without additional, laborious and time intensive treatments to obtain DNA suitable for PCR (8,13). Such treatments include the use of polyvinylpolypyrrolidone (PVPP), CTAB, hydroxyapatite columns, cesium chloride density centrifugations, size exclusion chromatography using Sephadex resins, and agarose gel electrophoresis followed by gel excision and extraction. In our experience, PVPP and CTAB have proven unreliable for the removal of inhibitors. Gel fractionation and Sephadex separation have been more reliable, but require additional time, cost and labor. Hydroxyapatite columns or cesium chloride density centrifugations are time-consuming and limit the number of samples that can be analyzed. Additionally, these procedures often result in decreased DNA recovery (11), possibly eliminating some target templates from complex communities.

To address these concerns, we collaborated with Mo Bio Laboratories, Inc. (Solana Beach, CA), in the design and testing of a simple and reliable kit for the isolation and purification of microbial DNA directly from soil. Our goal was a kit for small-scale sample processing that was suitable for field use with minimal equipment, and compatible with commercial machines such as Fastprep (Bio 101, Vista, CA) and the Mini-Bead Mill (BioSpec, Bartlesville, OK). The procedure used simple, proven methods in a five step process: lysis, protein precipitation, DNA binding, wash, and elution. The extraction utilized hot-detergent lysis in conjunction with mechanical homogenization. This combination has proven to be the most effective for lysing bacterial cells and endospores (6). We also developed a proprietary chemistry that removes DNA polymerase inhibitors during the lysis step, eliminating the need for additional treatments and the resuting loss of sample.

In this investigation, we tested the UltraClean™ Soil DNA Purification Kit, developed and sold by Mo Bio Laboratories, Inc. on a broad range of soils to evaluate the recovery and relative quality of microbial DNA. Soils were chosen to cover a wide range of sand/silt/clay compositions and were dry from long term storage. A hot detergent treatment was combined with vortexing to homogenize and lyse the soil microbes. The resulting DNAs were verified by gel electrophoresis and quantified by PicoGreen assay. The presence of inhibitors was detected by PCR using bacterial 16S rDNA primers. Amplification was verified by gel electrophoresis. Humic acid effects on PCR were also studied to determine the effectiveness of the Inhibitor Removal Solution (IRS) in a three phase trial. Humic acids were added to a non-inhibitory soil and the samples extracted using IRS in the lysis step. Next, soil samples that displayed inhibition after the standard extraction were re-extracted using IRS. Additional testing of the chemistry was conducted using peat bog samples, as they have proven to be a consistent challenge to microbial ecologists.

# Materials and Methods

## Soils

Samples were obtained from the soil collection of Dr. Tom Ruehr (Department of Soil Sciences, Cal Poly State University). Twenty-four soil types representing a wide range of sand/silt/clay composition were selected. The samples were stored dry (as received), at room temperature in sterile 50 ml Falcon tubes.

## Extractions

DNA extractions were performed using the UltraClean$^{TM}$ Soil DNA Kit (Mo Bio Labs, Inc). Samples were extracted in triplicate using 0.3 g of soil per replicate. The lysis step was modified to include a combination of hot detergent and mechanical lysis. Samples were first incubated at 70°C for 10 minutes. Mechanical lysis was conducted using a VortexGenie with a flathead by shaking the tubes horizontally at maximum speed for 10 minutes. The manufacturer's protocol was followed from this point.

## DNA Quantification

The yield and condition of the products was first estimated by agarose gel electrophoresis (1.5% agarose, TBE buffer, pH 8.25). The replicate DNAs from each were quantified with a 100µL PicoGreen (Molecular Probes, Eugene, OR) assay on a PE LS 50B luminescence spectrophotometer (PE-Biosystems, Foster City, CA) according to the manufacturer's protocols.

## PCR

DNA quality was determined directly by PCR. Templates were prepared by diluting the DNA samples 1:10 and 1:100 prior to PCR. Primers homologous to a 530 bp segment of the 16S rDNA were used for amplification. The sequences for the primers are as follows: K2R, 5'-GTA TTA CCG CGG CTG CTG C-3' and PAF, 5'-AGA GTT TGA TCC TGG CTC AG-3'. All PCR reactions were performed in a 50µL volume including 5µL of 10X reaction buffer (Promega, Madison, WI), 0.6 mM dNTP, 8 µg/µL bovine serum albumin, 2 mM $MgCl_2$, 2 U of Taq DNA polymerase (Promega), and 1µL of template. Reaction temperatures and cycling for the samples were as follows: 94°C for 2 min, 35 cycles of 94°C for 2 min, 48.5°C for 1 min, 72°C for 1 min, followed by a single cycle of 72°C for 10 min. Amplification was verified by electrophoresis using a 1.5% agarose gel in TBE buffer, pH 8.25.

## Soil Analysis

Total Carbon and Nitrogen analysis was kindly performed by the Shimel Lab at the University of California, Santa Barbara, using a Fisons Instruments NA 1500 Series 2 CN analyzer. Sand/silt/clay compositions were provided by Dr. Tom Ruehr (Department of Soil Sciences, Cal Poly State University).

## Epifluorescent Direct Counts

Microbial cell counts for each soil were estimated by epifluorescent direct count microscopy. Samples were initially diluted in 2.5% glutaraldehyde (GTA) in saline with 0.01 M tetrasodium pyrophosphate (NaPPi) and sonicated for 30 seconds. Serial dilutions were prepared with 2.5% GTA(saline. The diluted samples were stained with 100µl of 0.5 mg/ml DAPI. Stained samples were filtered through black polycarbonate membranes (0.2 µm pore size). Counts were made using an Olympus BX60F microscope with UV lamp. The theoretical yield of DNA was determined using the following formula:

$$\left(\frac{\text{Mean Cells}}{\text{Field}}\right)\left(\frac{\text{Field of View}}{\text{Area}}\right)\left(\frac{\text{Area of Filter}}{\text{Dilution}}\right)\left(\frac{\text{5e-9 µg DNA}}{\text{Cell}}\right) = \text{Theoretical DNA yield µg)}$$

## IRS Testing

Humic acids effects on the PCR were determined by adding known quantities of humic acid standards (Sigma Chemicals, St. Louis, MO) to 16S PCR amplifications. A side-by-side comparison using *E. coli* DNA and non-inhibitory soil DNA as templates was performed using the PCR conditions previously described. The effect of IRS on humic acids in soil was investigated by adding known quantities of humic acids to a non-inhibitory soil. The spiked soils were extracted by standard protocol using 200µl of IRS in the lysis step.

Problematic soils were re-extracted as previously described with the addition of 200µl of IRS following sample loading.

Peat bog samples were provided by Dr. Mark Schneegurt (Notre Dame). Peat samples were transferred to 1.5 ml microfuge tubes and centrifuge at 16,000 x g for 2 minutes to pellet cells and organic matter. The supernatant was decanted and 0.3 g of pellet was added to a lysis tube containing 200µl of IRS. Lysis was performed using a Fastprep instrument at 4.5 m $s^{-1}$ for 20 seconds. The samples were further processed as previously described.

# Soil Data

| Sample | Recovered DNA ng/ul | Recovered DNA ug/g | Theoretical DNA ug/g | Cells/g | % Carbon | % Nitrogen | % Sand | % Silt | % Clay |
|---|---|---|---|---|---|---|---|---|---|
| 9020 | 27.9 | 8.89 | 0.01 | 2.30E+06 | 0.81 | 0.10 | 64.2 | 25.7 | 10.1 |
| 9305 | 30.9 | 9.84 | ND | ND | 3.85 | 0.33 | 15.4 | 58.7 | 26.0 |
| 9307 | 19.5 | 6.20 | 0.01 | 2.78E+06 | 2.17 | 0.15 | 10.6 | 31.8 | 57.6 |
| 9308 | 23.1 | 7.36 | ND | ND | 4.66 | 0.33 | 67.6 | 27.3 | 5.1 |
| 9309 | 15.0 | 4.78 | 0.12 | 2.38E+07 | 0.97 | 0.10 | 13.7 | 66.5 | 19.8 |
| 9310 | 6.8 | 2.17 | 0.04 | 8.31E+06 | 3.51 | 0.31 | 29.7 | 53.3 | 17.0 |
| 9312 | 103.9 | 33.09 | 0.02 | 3.27E+06 | 2.58 | 0.22 | 15.9 | 66.1 | 18.1 |
| 9324 | 64.4 | 20.51 | 0.02 | 3.44E+06 | 1.18 | 0.08 | 36.8 | 54.1 | 9.2 |
| 9506 | 3.2 | 1.00 | 0.02 | 4.30E+06 | 1.56 | 0.11 | 35.6 | 49.0 | 15.5 |
| 9514 | 6.8 | 2.17 | 0.14 | 2.82E+07 | 3.20 | 0.19 | 23.4 | 46.3 | 30.3 |
| 9612 | 19.5 | 6.21 | 0.01 | 2.69E+06 | 1.61 | 0.15 | 25.7 | 54.6 | 19.8 |
| 9809 | 26.9 | 8.57 | 0.02 | 3.60E+06 | 1.28 | 0.14 | 26.0 | 44.8 | 29.3 |
| 9811 | 12.8 | 4.08 | ND | ND | 1.60 | 0.17 | 16.9 | 37.6 | 45.5 |
| 9813 | 16.3 | 5.19 | 0.02 | 3.33E+06 | 1.40 | 0.13 | 42.4 | 44.3 | 13.3 |
| 9818 | 30.2 | 9.62 | 0.01 | 2.08E+06 | 1.51 | 0.14 | 38.8 | 41.3 | 19.9 |
| 9822 | 21.4 | 6.82 | 0.09 | 1.82E+07 | 2.19 | 0.19 | 32.0 | 23.3 | 44.7 |
| 9824 | 15.4 | 4.91 | 0.12 | 2.43E+07 | 1.31 | 0.14 | 27.2 | 36.4 | 36.4 |
| 9834 | 25.0 | 7.96 | 0.02 | 3.53E+06 | 3.91 | 0.14 | 62.4 | 29.3 | 8.4 |
| 503515 | 0.6 | 0.19 | ND | ND | 0.82 | 0.14 | 50.0 | 35.0 | 15.0 |
| 851L | <0.2 | no value | ND | ND | 0.18 | 0.02 | 50.4 | 27.6 | 22.0 |
| 852L | <0.2 | no value | 0.01 | 1.88E+06 | 1.12 | 0.07 | 41.0 | 13.0 | 46.0 |
| 853L | <0.2 | no value | 0.01 | 2.28E+06 | 0.21 | 0.02 | 40.4 | 9.4 | 40.2 |
| N3 | <0.2 | no value | 0.01 | 2.12E+06 | 0.20 | 0.03 | 22.4 | 45.0 | 32.6 |
| San JD | 38.9 | 12.39 | 0.02 | 3.84E+06 | 19.44 | 1.37 | ND | ND | ND |

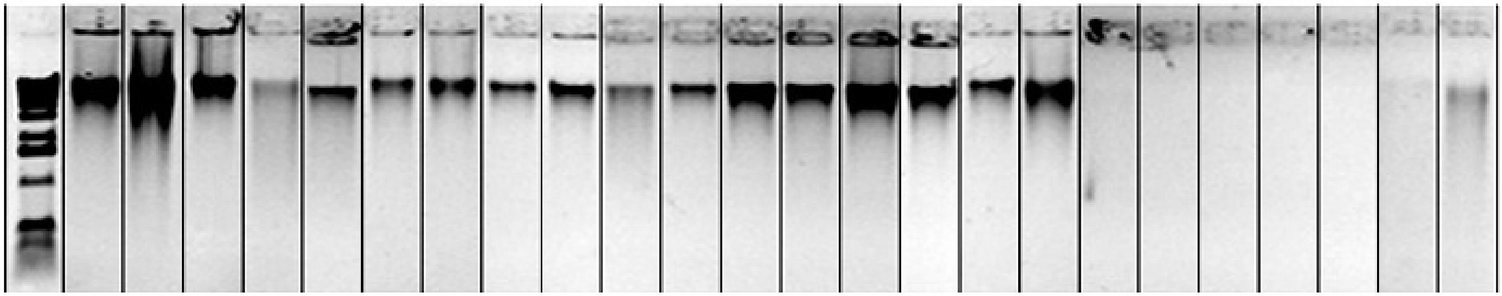
# Soil Data

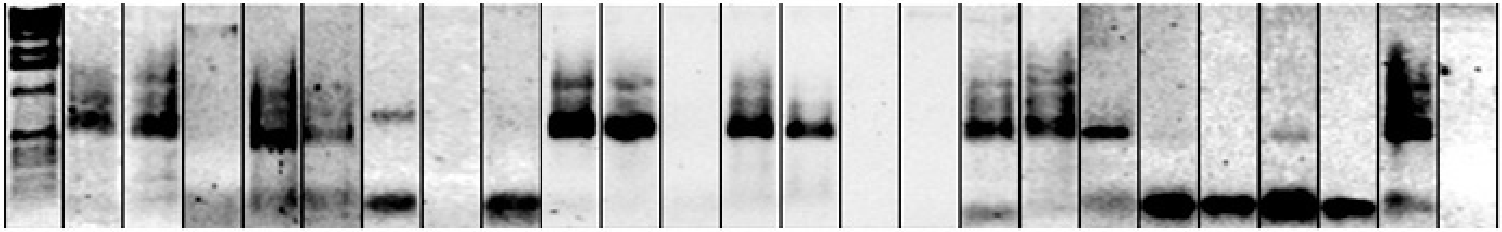
DNA yields were observed in soils with moderate carbon content (9310, 9312). However, low carbon soils produced very low DNA yields. The observed yields were lower than the range of 20 to 50 µg of DNA per gram of soil published previously (6), possibly due to the dessicated storage of our samples. Our results indicate the DNA yield from a given soil is largely independent of the carbon and nitrogen content, except in carbon-poor samples. The actual DNA yields were higher than the theoretical yields derived from direct count microscopy in all samples. This inconsistency is most likely due to the method used to release microbes from the soils. Previously, we optimized direct cell counts for low carbon-content, sandy soils using NaPPi with sonication. This method was not always effective in this study due to the diversity of soil types, but those similar to our sandy samples (carbon-poor) had the best correlation between cell count and DNA yield. Another problem encountered during this study was the effect of carbon content on staining efficiency. As carbon content increased, the dye concentration required to visualize cells also increased, likely due to the absorption of dye by organic compounds. Thus, each soil may require optimization of the dispersion method and staining in order to obtain accurate counts.

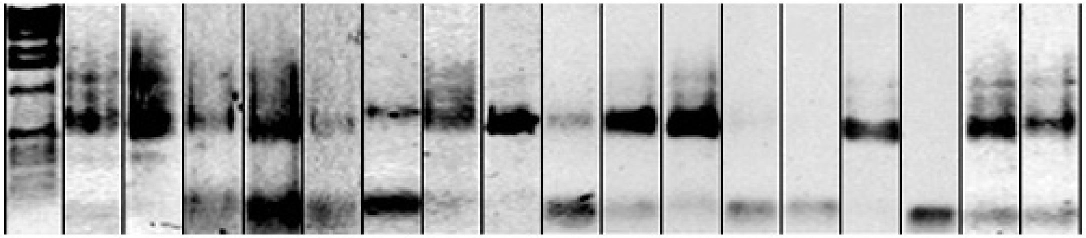
## Acknowledgments

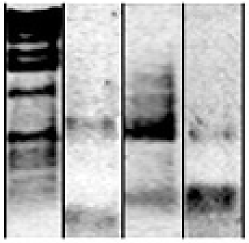
We would like to thank Dr. Tom Ruehr for providing the soils samples and sand/silt/clay data. We would also like to thank Dr. Mark Schneegurt for the problematic peats. And finally, we gratefully acknowledge LynnDee Althouse (Oyler) and the Schimel Lab at UCSB for the carbon and nitrogen analysis of the soils used in this study.

# 16S rDNA Amplification of Soil DNAs Without IRS









Row 1: Genomic DNAs isolated from soils
Row 2: PCR amplification of undiluted template DNAs
Row 3: PCR amplification of template DNAs diluted 1:10
Row 4: PCR amplification of template DNAs diluted 1:100

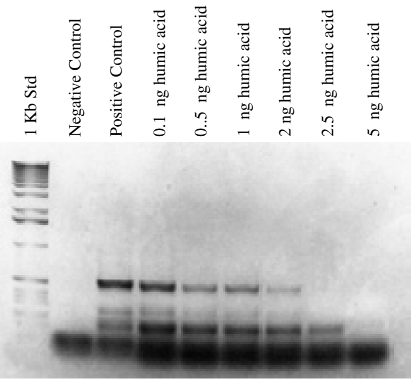
# 16S rDNA Amplification of Soil DNAs Without IRS

From the 24 soils extracted, 18 displayed visible genomic DNA on an agarose gel. PCR amplification of undiluted soil DNAs showed 14 of the 24 samples readily amplified without the use of IRS, three of which displayed no visible DNA in the initial analysis. Thus, 21 of the 24 samples yielded DNA (~88%), and 14 of the 21 (~67%) were suitable for PCR without additional processing.

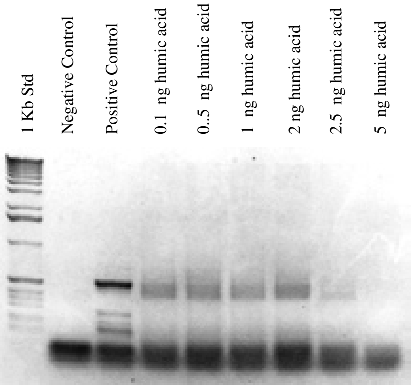
Inhibition was detected by the comparison of PCR products from diluted and undiluted template DNAs. Several parameters were used to define inhibition: no amplification at concentrations that were successful with other samples, no primer dimer formation, and primer dimer formation with no visible PCR product after dilution of the template DNA. We believe the last case is indicative of mild polymerase inhibition: the enzyme extends short segments and fails to amplify full length products. A total of seven samples (~33%) displayed inhibition and six of these were used to test the effectiveness of IRS. The seventh sample (9310) was not available in sufficient quantity for further experiments.

# IRS Testing

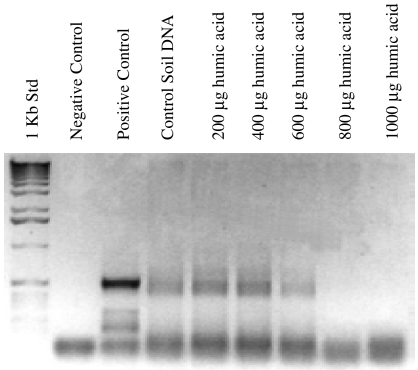
HUMIC ACID +*E. coli* DNA



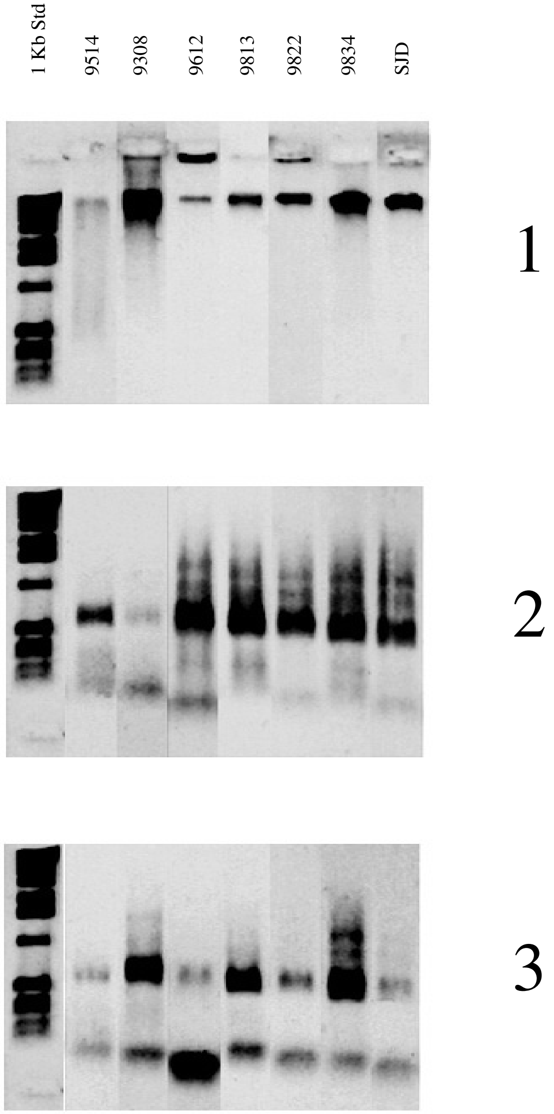
HUMIC ACID + SOIL DNA



SOIL + HUMIC FOR EXTRACTION



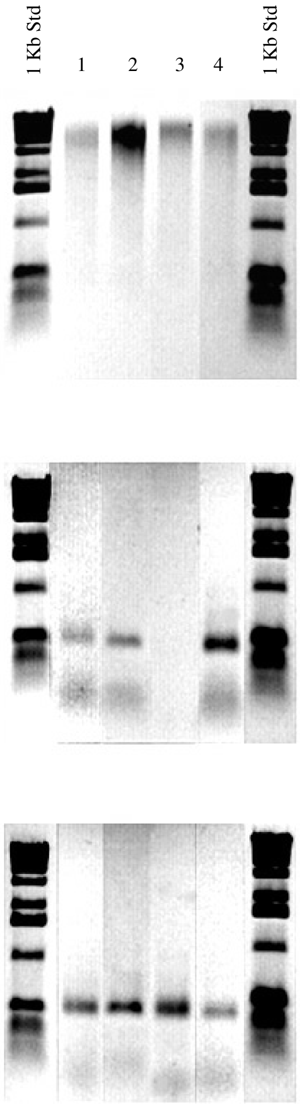
Soil DNAs Extracted With IRS



Legend for Soil and Bog Extractions

Gel 1: Sample DNAs
Gel 2: PCR of undiluted templates
Gel 3: PCR of templates diluted 1:10

Bog DNAs Extracted With IRS



Samples
1 - TB out - shore
2 - Fen Kickapoo
3 - Tender Boglake
4 - TB out 3.5

# IRS Testing

Humic acid effects on PCR were investigated by adding defined quantities of humic acids to PCRs. Both *E.coli* genomic DNA and extracted soil DNA were used as templates in separate trials. PCR with pure genomic DNA displayed inhibition after the addition of 2 ng of humic acid per reaction. Complete inhibition was observed with 5 ng of humic acid. The soil DNA displayed inhibition at 2.5 ng of humic acid per reaction, with 5 ng again producing complete inhibition. Thus, the PCR was completely inhibited when humic acids were present at a concentration greater than 50 pg/µl.

The Inhibitor Removal Solution (IRS) was tested by spiking a non-inhibitory soil (displaying no inhibition of PCR after normal processing) with known quantities of humic acid. A slight decrease in product was observed after spiking the soil with 600 µg of humic acid. Complete inhibition was apparent with 800 µg of added humic acid. The concentration of humic acid in the original sample was not known. The improved product quality in the 200 µgspiked sample over the control soil indicated some inhibitors were present. While the actual endpoint for this chemistry could not be precisely determined, it is apparent that the IRS does have a maximum binding capacity.

Samples displaying inhibition with the standard extraction were re-extracted using IRS in the lysis step. All soils produced DNA quantities comparable to the orignal extracts, though a slight reduction in yield was observed. This was due to increased volume in the lysis tube after the addition of the IRS. The standard volume of lysate was processed according to protocol and thus, some DNA was lost by dilution. Undiluted templates all produced visible PCR products after treatment with IRS. A control soil, Sample 9822, which displayed no signs of inhibition after the standard extraction, produced more PCR product when extracted with IRS. The San Joaquin Delta (SJD) soil displayed marked improvement. Without treatment, no amplification was observed in the undiluted template and only primer dimers formed in the diluted template, even though SJD produced the third largest DNA yield. After treatment, both the undiluted and diluted templates displayed products.

# Conclusions

We found the UltraClean™ Soil DNA Kit to be a simple and reliable, small-scale method for extracting and purifying DNA directly from-most soil types. In this study, the UltraClean™ Soil DNA Kit recovered DNA from samples using only standard laboratory equipment. There covered DNAs were suitable for PCR without the use of additional reagents in most cases. The Inhibitor Removal Solution (IRS) effectively removed known inhibitors such as humic acids from problematic soils without added time or labor.

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