Pre-PCR DNA quantitation of soil and sediment samples: method development and instrument design

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Abstract

A simple and straightforward method for the quantitation of dsDNA in soil and sediment matrices has been developed to support rapid, in-the-field PCR analysis of environmental samples. This method uses PicoGreen nucleic acid stain, and a combination of UV/Vis and fluorescence spectroscopy, to quantitate dsDNA in the presence of interfering humic materials. The practical utility of this approach is that it complements a seven-step DNA extraction procedure for environmental samples. The DNA quantitation method is utilized twice during the extraction procedure. Once, prior to a micro-spin column procedure to maximize the amount of DNA extracted, and a second time, just prior to PCR to optimize the PCR reaction conditions. A field-portable, assay-specific instrument has been developed based on this methodology. Software for this instrument steps the analyst through the experimental procedure, and has been designed such that a minimum of technical expertise is required to perform the assay. Initial data obtained from the prototype unit indicates that this instrument compares with commercial instrumentation in terms of detection limit and sensitivity. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Genotype analysis of soil DNA represents a considerable challenge for researchers involved in forensics, population genetics and microbial analysis of engineered microorganisms. Problems to overcome when pursuing these investigations include development of efficient DNA extraction procedures, and optimization of polymerase chain reaction (PCR) variables such that DNA from target organisms can be adequately amplified and identified. The ability to quickly and accurately quantitate low amounts of DNA in complex (interfering) matrices would be an asset to researchers involved with performing these types of analysis. UV absorbance ($A_{260}/A_{280}$ ratio) is commonly used to determine nucleic acid concentration. However, quantitation of low DNA concentrations by this method often proves unreliable due to its limited sensitivity and susceptibility to interference (Ahn et al., 1996; Singer et al., 1997a). Fluorescence-based methods also exist, though fluorescence quenching from naturally occurring compounds present in environmental samples hinders this approach as well (Sandaa et al., 1998).

DNA extracted from environmental samples also contains many contaminants that inhibit PCR assays (Jacobsen, 1995; Moreira, 1998). Predominant among these are humic substances present in virtually all samples containing soil-derived organic material. It has been documented that humic materials inhibit Taq polymerase (Tsai and Olson, 1992a), and decreases effi-
ciencies of DNA-DNA hybridizations (Tebbe and Vahjen, 1993). Further inhibition has been reported through binding of PCR primers and chelation of Mg\(^{2+}\) ions by humic substances (Tsai and Olson, 1992b). Humic materials have reportedly been removed from DNA samples using a Sephadex G200 spin column procedure (Tsai and Olson, 1992b). However, success with this method is dependent on the amount of DNA purified through a single column. We have found purification to be much more efficient and successful if one can estimate the amount of DNA loaded on to the purification column (Kuske et al., 1998).

DNA concentrations in crude samples are commonly estimated by placing an aliquot of the material on an agarose gel, running the gel, then staining with ethidium bromide (EtBr). The procedure requires time to prepare and run, and a UV light source equipped with photography equipment to visualize the results. Further, the optimal dye-to-base pair ratio is extremely narrow for EtBr and accurate measurements are possible only within a narrow range of concentrations (Singer et al., 1997a). Therefore, the agarose gel technique is impractical for high throughput DNA quantification.

Recently, we developed and optimized preparative procedure for the recovery of DNA from environmental sample types such as soils, sediments, and tissues for subsequent PCR analysis (Kuske et al., 1998). It has been determined that DNA quantitation is critical at two points in the extraction procedure. The first quantification step is necessary prior to a micro spin-column procedure whose purpose is to remove humic materials and other contaminants known to inhibit PCR. This is essential because the effectiveness of spin columns to remove humic materials is highly dependent on the concentration of DNA extracted. That is, sample flow and clean up through the cartridges is optimal within a specific range of DNA concentrations and sample volumes.

The second DNA quantification step, performed at the end of the extraction procedure and just prior to PCR, is necessary because PCR is enzyme-catalyzed, and as such is mediated by reaction kinetics (Morrison and Gannon, 1994; Suzuki and Giovannoni, 1996). Non-target DNA at concentrations greater than 1 mg/mL have also been reported to inhibit PCR (Jacobsen and Rasmussen, 1992). Therefore, prior knowledge of the amount of DNA in the sample ahead of time allows the proper reaction stoichiometry to be optimized, permits the highest sensitivity PCR to be performed, and greatly decreases the possibility of obtaining false negatives.

The successful method development and instrument design capable of performing the two pre-PCR DNA quantification steps just described, would contribute to many areas that utilize genetic analysis techniques. Military operations, focusing on the detection of biological warfare agents and utilizing micro PCR devices, have an interest in a detection system as described. Hazardous Material Response Units such as the FBI and forensic laboratories can incorporate such a device as part of newly conceived “fly-away” mobile laboratories now under development. Commercial and governmental civilian laboratories such as the United States Department of Agriculture benefit, for example, for use in pathogen detection for meat packaging operations.

The work reported here is part of a larger on-going effort in which the programmatic objectives are to develop reliable methods for the extraction of DNA from a diverse range of environmental sample types to support rapid in-the-field PCR analysis. This article details the DNA quantification component of the extraction procedure optimized for the extraction and purification of DNA from soils and sediments (Kuske et al., 1998). We were faced with three specific tasks in this regard: (1) develop a sensitive and reliable assay for the quantitation of dsDNA in the presence of interfering humic materials, (2) develop a field-portable instrument capable of performing the analysis, and (3) insure that the method and instrument should be user-friendly and as automated as possible.

2. Materials and methods

Intercalating nucleic acid stains ethidium homodimer-1 (EthD-1), ethidium homodimer-2 (EthD-2), POPO-3, PO-PRO-3, TO-PRO-1, TOTO-1, YO-PRO-1, and PicoGreen were obtained through Molecular Probes Incorporated (Eugene, OR http://www.probes.com). These dyes were prepared and used according to manufacturer recommended procedure. Promega Lambda DNA was purchased through Fisher Scientific (Pittsburgh, PA 412-562-8300). Tris acetate EDTA buffer (TAE, pH 8.2) was prepared in house from reagent grade chemicals using distilled deionized water. For DNA quantification using PicoGreen, samples and standards in TAE buffer were mixed with equal volumes of diluted PicoGreen reagent (1:200 in TAE). DNA calibration standards were prepared using lambda DNA at final assay concentrations of 0.1, 1.0, 5.0, and 10.0 ng Ml\(^{-1}\). After the dye was added, all samples were allowed to equilibrate under gentle agitation using a Labquake tube shaker (Labindustries, Berkeley, CA 510-843-0220) for 10 min in the dark. Humic materials were obtained through Sigma (St. Louis, MO 314-771-5750), from the International Humic Substance Society (Department of Soil, Water, and Climate, University of Minnesota, St. Paul, MN 612-625-4711), or were isolated from Colorado peat (Paul Brinkerhoff, Salida, Colorado). Briefly, the
humic and fulvic extraction from the Colorado peat involved sieving the peat to a 150–70 μm (100/200) mesh size followed by 8 h of alkali extraction under a nitrogen atmosphere. The soluble fraction was collected and was acidified to a pH of 1.5. The precipitant (isolated humic acid) was removed from the supernatant (the fulvic acid) and both were lyophilized to dryness. Fluorescence measurements were either made on a Perkin-Elmer LS-5 laboratory fluorometer (Norwalk, CT 800-762-4000), or a SPEX Industries Fluorolog-3 research grade fluorometer (Edison, NJ 800-438-7739) using a standard 1-cm pathlength quartz fluorescence cuvette. The excitation and emission wavelengths were set to correspond to the maximum absorption and emission wavelength of the dye under investigation, which were determined experimentally. The excitation and emission slits were set to a 5-nm bandpass unless otherwise noted. UV/Vis absorption measurements were done on a Hewlett Packard 8452A scanning diode array spectrophotometer (Palo Alto, CA 800 227-9770) also in a standard 1-cm quartz cuvette. Any necessary dilutions were performed using TAE buffer. The amount of DNA present in the assay samples was calculated based upon linear interpolation against known DNA standards. Reagent control (no DNA) background fluorescence was subtracted from calibration and assay samples.

3. Results and discussion

3.1. Method development

Several commercial intercalating dyes (EthD-1, EthD-2, POPO-3, PO-PRO-3, TO-PRO-1, TOTO-1, YO-PRO-1, and PicoGreen) were evaluated in terms of their fluorescence characteristics, chemical and photochemical stability, and their ability to detect dsDNA in solution (experimental data not shown). Literature articles were also reviewed to gain further information on these dyes with regards to reported detection limits and interferences. A summary of the results from these studies is presented in Table 1. PicoGreen was determined to be the best candidate in which to focus our research efforts. This dye demonstrated superior detection limits, the greatest linear dynamic range, and was easy to prepare and use. An overlay of the respective UV/Vis absorption spectra obtained from 25 μg ml⁻¹ aqueous solutions of humic and fulvic acids obtained from Colorado peat and from the International Humic Substance Society is shown in Fig. 1. Each of the materials examined show relatively featureless absorption spectra with absorptivities increasing towards the shorter wavelengths. These spectra offer little information regarding chemical structure although the likeness of the spectra among
the group as a whole suggests that there is a similarity in the type of compounds present in each of the respective samples. This figure is intended to show the similarity of these materials resulting from UV/Vis interrogation.

The interfering effects that humic materials have on DNA quantitation are presented in Fig. 2a and 2b. As small amounts of humic acid are added, a clear attenuation in the fluorescence signal is observed. The lower curve in Fig. 2a represents the greatest attenuation and demonstrates the effect that as little as 5 \( \mu g \) ml\(^{-1} \) humic acid has on the quantitation of DNA in solutions containing these materials. This interference is primarily attributed to fluorescence quenching by soil-derived organic substances (Sandaa et al., 1998). In addition, intrinsic fluorescence of humic materials (nominally excited at the excitation wavelength for PicoGreen), is also troublesome. Background intensity increased (Fig. 2b) as the concentration of organic matter was increased. Consequently, low amounts of DNA present in these matrices were difficult to detect and quantify. Further, interference likely occurs due to phenolic residues present in humic materials, which have been reported to interfere with the intercalating action of these types of dyes (Ahn et al., 1996; Singer et al., 1997a).

A sample dilution method has been reported to overcome fluorescence quenching due to the presence of interfering humic materials in soil extracts (Sandaa et al., 1998). In order to ensure an accurate analysis, standard additions involving serial dilution procedures are required to ascertain the correct value for the DNA present in the sample. These procedure can be laborious and time consuming, and are not conducive to field-portable or high throughput sample analysis.

We have also found that by diluting crude soil extracts, reliable quantitation of remaining DNA in these samples can commence using PicoGreen nucleic acid stain. To obtain accurate results though it is essential to determine the correct dilution level to prevent attenuation of the fluorescence signal. Fig. 3a shows the serial dilution of three different soil extracts with the DNA quantification results plotted as a function of the dilution factor used for the analysis. Sample 1 was extracted from an Arizona soil, sample 2 from an Ohio soil, and sample 3 was from a New Mexico soil. The extraction procedure, soil texture, chemical composition, and organic matter content were described by Kuske et al., (1998). At a dilution level of 1000 and contact with PicoGreen nucleic acid stain, fluorescence signals were obtained for each sample. If one was not aware there was significant fluorescence quenching occurring due to the presence of residual humic compounds, those results might be reported. Only after various dilutions have been analyzed for each sample, and the respective DNA values have leveled off for consecutive dilution levels, can valid DNA concentrations be reliably obtained. In Fig. 3a, sample 2 levels off at a dilution of roughly 2500 (14.8 \( \mu g \) DNA ml\(^{-1} \)), sample 3 at approximately 5000 (9.1 \( \mu g \) DNA ml\(^{-1} \)), and sample 1 (the most extreme case) at a dilution of 10,000 (76.9 \( \mu g \) DNA ml\(^{-1} \)).

The UV/Vis spectrum for sample 1 was tracked as a function of the dilution level required to overcome the humic material interference (Fig. 3b). A maximum of 0.05 absorbance units (occurring at ca. 240 nm) is tolerated before significant interference by the humic materials present in the sample manifest themselves. Similarly, at a 2500-fold dilution, sample 2 had a

![Fig. 1. UV/Vis absorption spectra of 25 \( \mu g \) ml\(^{-1} \) isolated humic fractions from different origin at pH 7.5. Curves 1 and 2, respectively, refer to isolated fulvic and humic acid extracted from Colorado peat. Curves 3 and 4, respectively, are fulvic and humic acid reference standards obtained from the International Humic Acid Society.](image-url)
Fig. 2. (a) PicoGreen/DNA fluorescence intensity for a series of solutions containing from 1–10 ng lambda DNA ml\(^{-1}\), in the presence of varying amounts of humic acid. Sigma humic acid concentration has been varied from 0 to 5 \(\mu\)g ml\(^{-1}\) in TAE buffer. Significant quenching occurs as evidenced by decrease in slope of calibration curves as humic acid concentration is increased. Fluorescence measurements were made with excitation at 502 nm and the resulting emission monitored at 525 nm. Background fluorescence resulting from intrinsic humic acid fluorescence at these wavelengths has been subtracted. (b) The change in fluorescence background as a function of increasing humic acid concentration is shown. Experimental conditions were as described in Fig. 2a. Background fluorescence resulting from intrinsic humic acid at these wavelengths has not been subtracted.
maximum absorbance of 0.06 AU and sample 3 exhibited a maximum absorbance of 0.04 AU at a 5000-fold dilution (data not shown). To corroborate the initial observation that humic material interference is eliminated at these absorbance levels, 22 samples of known DNA concentrations, with varying amounts of humic acid (500 –1000 μg ml⁻¹), were prepared. The experimental procedure was to first dilute each sample in 1/10 TAE buffer until the maximum absorbance occurring between 220 and 400 nm did not exceed 0.05 absorbance units for each sample. Once an adequate dilution had been determined for each sample submitted, a fluorescence-based DNA quantitation step was performed using PicoGreen dsDNA quantitation reagent.

The assay values track very nicely with the known or target values (Fig. 4). Moreover, the UV/Vis absorption pre-screening step was successful in eliminating the interference caused by the humic materials present in the sample. The average deviation of the assay value compared to the known or target values was calculated to be ±3.7%. The linear regression line ($R^2 = 0.997$) was nearly identical to the 1:1 line. The UV/Vis pre-screening step is

![Graph](image)

Fig. 3. (a) Effect of sample dilution on DNA quantitation in the presence of soil organic matter. The interfering effects of humic materials are eliminated when an adequate dilution has been attained as evidenced by consistent quantitation results for successive dilutions. Sample 1 had the greatest humic content (2.75%). (b) The UV/Vis absorption spectra is tracked as a function of the dilution level required to overcome the humic material interference. As indicated from Fig. 3a, a 1:10000 dilution is required for this sample, corresponding to a maximum humic material absorbance of 0.05.
successful for indicating the correct dilution for subsequent fluorescence measurements because humic materials have similar absorption characteristics. Further, it has been reported that fluorescence quenching from residual organic matter is eliminated at low concentrations (Sandaa et al., 1998). Experiments in our laboratory have confirmed that at humic concentrations below 500 mg ml\(^{-1}\), monitored at the peak excitation and emission wavelengths for PicoGreen nucleic acid stain, intrinsic fluorescence from residual humic materials is not detected (data not shown). This eliminates the need for fluorescence background subtraction due to the organic matter present in these samples, when they have been prepared according to this experimental procedure.

The spectroscopic (UV/Vis absorption, fluorescence excitation and emission) properties of the key constituents involved when performing DNA analysis in soil and sediment-derived samples are presented in Fig. 5. This overlay shows that by choosing a fluorescent tag which falls within the Stoke’s shift of the major contaminant, and by minimizing the interfering effects of the humic materials through dilution, that DNA can be reliably detected in matrices of this type using the method we have described. It is not practical to report a detection limit for this method because that will depend upon the concentration (and required dilution) of the interfering material present in the sample matrix. In the absence of any humic material, the lower limit of detection of DNA using PicoGreen Dye has been reported to be 25 pg DNA ml\(^{-1}\) (Ahn et al., 1996; Singer et al., 1997a).

### 3.2. Instrument development

Our next task involved the design of a prototype instrument that incorporated the DNA quantitation method in a field-portable and assay-specific unit. These efforts lead to the construction of an instrument for quantifying DNA in samples that contain interfering humic materials, which incorporates both UV/Vis absorption and fluorescence modes of detection.

A schematic representation of the developed portable DNA quantifier (PDQ) is shown in Fig. 6. The PDQ is powered by a single 12-V battery and housed in two (28 × 14 × 17 cm) modules. The combined weight of both units is approximately 6 kg. The instrument control module contains the user interface, data management, and test control functions. Within the measurement module are the sample chamber, light source and detection electronics, and the optical components necessary to perform the analysis. A modulated xenon light source (Hamamatsu Photonics Systems, Bridgewater, NJ 908–231–1116) is used for both absorption and fluorescence modes of analysis. Focusing lens one (FL1) collects the output of the xenon lamp and focuses it onto a standard 1-cm quartz sample cuvette. When the PDQ is operating in the absorption mode, focusing lens two (FL2) collects the transmitted light that has passed through the sample and directs it into a fiber optic assembly, 180° from the xenon light source. From there, it is coupled into a Carl Zeiss miniature spectrometer (model MMS-UV, Hellma Incorporated, Forest Hills, NY 718-544-9534) which covers the 190–400 nm range of

![Fig. 4. DNA quantitation results from 22 samples comparing expected versus measured values of DNA in varying concentrations of humic acid. Samples were prepared with known amounts of Promega Lambda DNA and Sigma humic acid. Prior to fluorescence analysis, samples were pre-screened by UV/Vis analysis to ascertain a dilution level corresponding to 0.05 maximum humic material absorbance.](image-url)
the electromagnetic spectrum. The separated light is detected with a 256-element diode-array that comes attached to the miniature spectrometer.

To perform fluorescence readings, a 12-V solenoid actuates a 485-nm cut-off filter that is out of the optical path for the absorption measurement, but moves between the sample cell and the xenon lamp for the fluorescence measurement. This filter absorbs electromagnetic radiation below 485-nm to minimize excitation of other fluorophores in solution (e.g., humic materials). The PicoGreen/DNA complex is excited at 502-nm and the resulting fluorescence is measured at 90° by a R1477 photomultiplier tube (Hamamatsu Photonics Systems, Bridgewater, NJ 908-231-1116) after first passing through a 535-nm notch filter. This allows detection of the 535-nm fluorescence resulting

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**Fig. 5.** Normalized key constituent spectral properties for the analysis of dsDNA in soil and sediment samples. $FA_{abs}$, $HA_{abs}$, and $DNA_{abs}$ correspond to fulvic acid, humic acid, and DNA absorption spectra. $HA_{EX}$ and $PG_{EX}$ refer to humic acid and PicoGreen fluorescence excitation spectra. $PG_{EM}$ and $HA_{EM}$ correspond to PicoGreen and humic acid fluorescence emission spectra.

**Fig. 6.** Schematic representation of the portable DNA quantifier (PDQ) designed for DNA quantitation in soil and sediment samples.
from the labeled DNA, and prevents background interference originating from the xenon excitation source from reaching the photomultiplier tube.

The developed analytical method was coded as an internal algorithm and incorporated into the prototype PDQ design software. After an initial absorbance reading, this routine instructs the operator as to the proper dilution of the sample to overcome humic material spectral interference. Data is input into the PDQ either through one of the eight softkeys, or through a thumbwheel, located on the front of the instrument control module. An RS-232 port supports output to a printer, computer, and a 3.5-inch disk drive for subsequent data storage and retrieval.

3.3. Prototype evaluation

A comparative test was performed in which 15 soil samples of unknown DNA and humic material content were subjected to the developed analytical procedure. The extraction procedures utilized for these samples have been described by Kuske et al. (1998). The DNA analysis was performed using conventional laboratory instrumentation (a Hewlett Packard 8452A spectrophotometer, a Spex Industries Fluorolog-3 Fluorometer) and by the PDQ custom spectrophotometer. There was good agreement (Fig. 7) between DNA concentrations obtained by commercial instrumentation and the prototype PDQ. A t-test was performed on the individual differences between results for each sample. For each soil sample, there were no significant differences in estimated DNA concentration obtained by the PDQ and current lab based instrumentation chosen to perform the analysis. Four replicate measurements were performed for each sample by the PDQ, with the average standard deviation calculated to be 1.06 % of the mean reading for each sample.

4. Conclusions

A straightforward analytical method has been demonstrated for the quantitation of dsDNA in soil and sediment samples. This method uses UV/Vis absorption spectroscopy to first prescreen samples to ascertain an adequate dilution level to just overcome the effects of interfering humic materials. This procedure frees the investigator from performing serial dilutions when performing DNA analysis in these types of matrices. A field-portable custom spectrophotometer has been constructed for the developed assay that compares with bench top instrumentation in terms of sensitivity and accuracy.

Using the PDQ in combination with the small-scale extraction procedure optimized by Kuske et al. (1998), allows in-the-field sample preparation of complex samples for genetic analysis. False positives are greatly reduced through the elimination of multi-step extraction techniques, which helps prevent cross contamination among samples. DNA extraction efficiency is optimized with DNA quantification performed on crude soil extracts before employing sample clean up.
procedure. Additionally, false negatives are reduced by using the PDQ to quantitate DNA just prior to PCR. This allows the reaction conditions to be optimized and permits the highest sensitivity PCR to be performed.

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References


