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A Simple, Efficient Method for the Separation of Humic Substances and DNA from Environmental Samples

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Three different gels (Sepharose 4B, Sephadex G-200, and Sephadex G-50) were evaluated as a means of removing humic contaminants from DNA extracts of environmental samples. Sepharose 4B gave superior separation of DNA from humics, and DNA purified in this way showed consistently greater amplification than DNA purified by the other materials.

Molecular analyses of natural microbial communities are often dependent upon the extraction and purification of nucleic acids. This dependence applies especially to procedures involving the amplification of regions of DNA via PCR, which is particularly sensitive to contamination by humic substances (16). While various methods can be used to extract crude nucleic acids from soils and sediments, purification of these samples to obtain high-quality DNA is the rate-limiting step in most studies, and many of the modifications to existing extraction methods have been developed to minimize humic contamination (1).

Most methodologies for separation of DNA from humic materials are dependent upon either differential levels of binding of humic substances and nucleic acids to a polymeric matrix or differential size fractionation (1, 6–8, 12–14, 21). These methods vary in their rapidity and ease of use, as well as in their effectiveness. One of the most widespread techniques is the use of spin columns packed with various matrices (3, 9, 17). Typically, the separation of DNA and humic substances through the use of spin columns is fast and relatively inexpensive, but the degree of separation is dependent upon the nature of the packing material (17). One commonly used packing gel is Sephadex G-200 (2, 5, 17), which usually removes humic substances sufficiently to yield DNA that is pure enough for amplification, although further dilution may be required (17). Here, we describe the use of a gel matrix developed for the separation of nucleic acids and polysaccharides in the purification of DNA from a variety of environmental samples. This matrix improves upon the separation of humic substances and DNA achieved with Sephadex G-200 and significantly increases the efficiency of subsequent amplifications.

Four environmental samples were used: woodland soil, hydrocarbon-contaminated soil, aquatic sediment, and an aquatic biofilm. Soil was obtained from a wooded lot on the campus of the University of Alabama, Tuscaloosa, Ala. Contaminated soil was produced by exposing a subsample of the woodland soil to 400 mg of diesel fuel g^{-1} for 12 weeks. Aquatic sediment was taken from a small wetland in Hale County, Alabama. The biofilm sample was taken from the surfaces of leaves of white water lilies (*Nymphaea odorata*) growing at the same site. Similar quantities of each sample (2 g [wet weight]) were used.

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DNA was extracted from each sample by the high-salt, sodium dodecyl sulfate-based, extended-heating method of Zhou et al. (22). Following centrifugation (6,000 × *g*, 10 min), the supernatant was collected and nucleic acids were extracted with phenol-chloroform (1:1), precipitated in alcohol overnight (11), and resuspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Crude nucleic acid extracts from all samples had a yellow-brown color.

Spin columns were constructed by plugging 1-ml plastic syringes with 1 cm of glass wool and packing the syringes with Sepharose 4B (Sigma). Syringes were centrifuged (1,100 × *g*, 5 min) and repacked following compaction. Centrifugation and repacking continued until the syringe contained approximately 4 cm of gel matrix, whereupon the columns were washed with TE buffer and excess buffer was removed by a further centrifugation. Other spin columns containing Sephadex G-200 or G-50 (Sigma) were manufactured similarly.

Aliquots (100 μl) of each sample were loaded onto each of the three column types, and the eluents were collected after centrifugation (1,100 × *g*, 5 min). All columns were then loaded with 100 μl of TE buffer and spun again, and the eluent was collected. Washing with TE was continued until visible signs of humic contamination (typically a brown band) had passed through the columns. The optical density at 260 nm (OD_{260}) of each fraction was determined. Fractions from the spin columns were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and photographed. The quantities of DNA eluted in the fractions were compared by examining the relative intensities of bands as determined from digital photographs obtained with NIH Image software, version 1.54 (by Wayne Rasband, National Institutes of Health, Bethesda, Md.; available from FTP:zippy.nimh.nih.gov).

Originally, we intended to use OD_{260} as a measure of DNA concentrations but found OD_{260} to be a truer indication of levels of humic substances. There was no relationship between DNA concentrations determined from agarose gels and OD_{260} (Fig. 1), as others have observed (13). Similarly, OD_{280} and $OD_{260/280}$ did not correlate with DNA concentration and were more dependent upon the presence of humic materials within the sample (data not shown). Quantification of DNA by other spectrometric methods was not possible because of humic contamination in many fractions, particularly those eluted from Sephadex G-200 and G-50 columns.

The degrees of separation of nucleic acids from humic substances varied depending upon the column matrix (Fig. 1). Sepharose 4B gave the best separation of the two substances

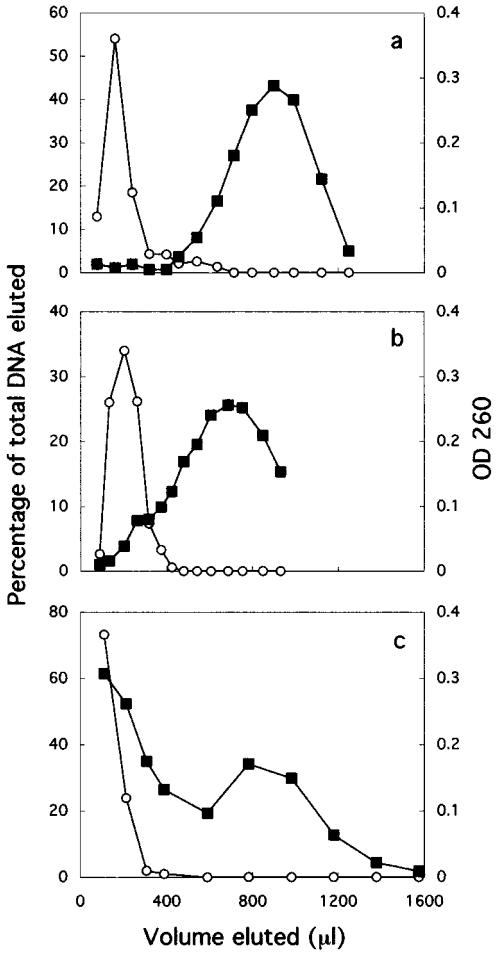

FIG. 1. Separation of DNA (as quantified from agarose gels, open circles) and humic material (as determined from OD_{260}, filled squares) extracted from woodland soil by spin columns containing different materials: Sepharose 4B (a), Sephadex G-200 (b), and Sephadex G-50 (c). Each point represents a separate fraction obtained after the sample was washed with 100 μl of TE buffer. Similar results were obtained for samples from contaminated soil, wetland sediment, and a wetland biofilm.

for all samples. Sephadex G-200 was less effective, with detectable quantities of humic substances being present in early fractions that also contained significant concentrations of DNA (Fig. 1b). As others have shown (17), Sephadex G-50 was ineffective at purifying DNA from environmental samples (Fig. 1c). Thus, yields of purified DNA were typically greatest with Sepharose 4B and least with Sephadex G-50.

For each sample, the fraction that contained the greatest concentration of DNA from each column was selected for amplification. Samples were diluted to give equal concentrations of DNA in each, and the variable V3 region of 16S DNA coding for rRNA was amplified with the primers of Muyzer et al. (10). Amplification was performed in 50-μl capillary tubes with a Rapidcycler (Idaho Technologies) according to the manufacturer's specifications (18–20). Final reaction mixtures contained 0.4 μM each primer, 0.2 mM each deoxyribonucleotide triphosphate, 50 mM Tris-HCl (pH 8.3), 0.25 mg of bovine serum albumin per ml, 3.0 mM $MgCl_2$, and 1.0 U of AmpliTaq polymerase (Perkin-Elmer). We used a touchdown procedure (4) and started with an annealing temperature of 65°C for 4 cycles. The temperature was then lowered to 63°C for 4 cycles and subsequently decreased by 2°C every second

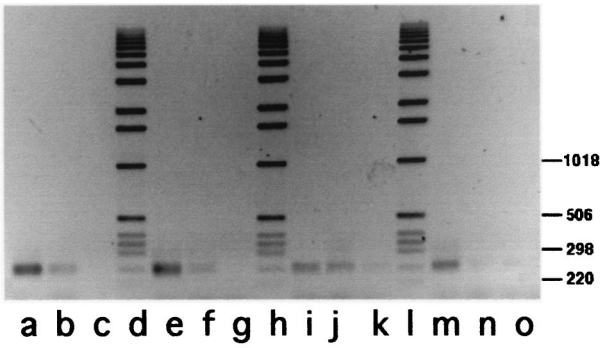

FIG. 2. PCR amplifications of DNA from environmental samples purified through various spin columns. Lanes: a, woodland soil through Sepharose 4B; b, woodland soil through Sephadex G-200; c, woodland soil through Sephadex G-50; e, contaminated soil through Sepharose 4B; f, contaminated soil through Sephadex G-200; g, contaminated soil through Sephadex G-50; i, wetland sediment through Sepharose 4B; j, wetland sediment through Sephadex G-200; k, wetland sediment through Sephadex G-50; m, biofilm through Sepharose 4B; n, biofilm through Sephadex G-200; o, biofilm through Sephadex G-50. Lanes d, h, and l contain 1.0 kb of Gibco-BRL marker DNA (numbers at the right denote length in base pairs). The photograph was scanned through Adobe PhotoDeluxe 1.0.1 with a PowerPC Macintosh.

cycle until we reached a touchdown at 55°C, where a further eight cycles were carried out. Amplification products were analyzed by agarose gel electrophoresis, and band intensities were quantified with NIH Image.

For each sample, the DNA purified with Sepharose 4B columns, when amplified, produced distinctly more product than DNA purified with Sephadex G-200 columns (Fig. 2). Aquatic samples processed with Sephadex G-50 showed some amplification, but the products were barely detectable (Fig. 2). Similar results were obtained from three replicate amplifications.

While the Sepharose 4B spin columns yielded DNA that was virtually free of humic contamination, this result may have been dependent upon the particular extraction method used (22). However, we have also used the Sepharose 4B columns to purify DNA obtained by other methods (freeze-thaw and lysozyme incubation [15]) with the same results (data not shown). The large pore size of Sepharose 4B also removes much low-molecular-weight RNA from nucleic acid extracts, further purifying the eluted DNA.

Sepharose 4B spin columns have been shown to be capable of purifying DNAs from a variety of aquatic and terrestrial habitats. The manufacture of these columns is simple and fast (typically, 12 or more columns can easily be produced in an hour). Columns can be prepared in advance and refrigerated prior to use. This approach should be suitable for use in any procedure requiring highly purified DNA from environmental samples.

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