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Note

Evaluation of Methods of Determining Humic Acids in Nucleic Acid Samples for Molecular Biological Analysis

Yong WANG and Takeshi FUJII[†]

National Institute for Agro-Environmental Sciences, 3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604, Japan

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It is important in molecular biological analyses to evaluate contamination of co-extracted humic acids in DNA/RNA extracted from soil. We compared the sensitivity of various methods for measurement of humic acids, and influences of DNA/RNA and proteins on the measurement. Considering the results, we give suggestions as to choice of methods for measurement of humic acids in molecular biological analyses.

Key words: humic acids; metagenome; soil; spectroscopy

Humic acids, as rich organic constituents of soil, often appear as impurities in the nucleic acids (DNA or RNA) extracted from soil for molecular biological analysis, as in metagenomic study. In order to evaluate the quality of the extracted DNA/RNA, several methods for determining concentrations of co-extracted humic acids were developed. These methods were divided into three types, as follows: visual colorimetry,[1)] visible and ultraviolet spectroscopy,[2–5)] and fluorescence spectroscopy,[1,4,6)] but information on important features of most of the methods, such as detection limit, linear range, and disturbing substances, is not available. Thus, it is unclear under what conditions these methods are suitable to determine the concentration of co-extracted humic acids in nucleic acids extracted from soil. In this study, we compared the sensitivity of the methods to each other for measurement of humic acids using a commercial humic acid derived from soil, and the threshold concentrations of the nucleic acids and protein molecules affecting the measurement of humic acids by the various methods. Considering the results obtained, we give suggestions as to the conditions under which these methods should be used.

A NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an F-2500 Fluorescence Spectrophotometer (Hitachi, Tokyo) were used to determine the concentration of humic acids, as previously described.[1)] A commercial humic acid (Nacalai Tesque, Kyoto), originating in soil, was dissolved in 0.1 M NaOH. After brief centrifugation to remove undissolved materials, the humic acid solution was diluted serially with Milli-Q water (Millipore, Billerica, MA). The spectroscopic characteristics of the commercial humic acid are shown in Fig. 1A and B, and suggest that it possesses typical spectroscopic characteristics for humic acids extracted from soil. The detection limit of the visual colorimetry method was determined by comparing a set of serial diluted humic acid solutions with water (Fig. 1C). To determine the linear range of each spectroscopic method, serially diluted humic acid solutions in triplicate (from 0.1 ng/μL to 1 μg/μL for visible and ultraviolet spectroscopy, and from 0.01 ng/μL to 20 ng/μL for fluorescence spectroscopy) were determined by each method. The linearity of the data was tested by squared correlation (R^2) on Microsoft Excel. Disturbance of DNA, RNA, and protein was measured by comparing the fluorescence intensities of DNA, RNA, BSA (bovine serum albumin) or skim milk at different concentrations with those of the humic acids. The concentration of DNA, RNA, BSA or skim milk corresponding to the signal intensity lower than the lower limit of the linear range of humic acids detection was considered to represent no effect on the determination of humic acids. Genomic DNA and total RNA were extracted from the *Pseudomonas putida* KT2440 strain with a Puregene DNA Purification Kit (Gentra, Minneapolis, MN) and an RNeasy Mini Kit (Qiagen, Valencia, CA) respectively. The genomic DNA or total RNA was mixed with different amounts of the commercial humic acid prior to real-time PCR or real-time RT-PCR. The abundance of the genomic DNA and RNA of the 16S rRNA gene in *P. putida* KT2440 was examined by real-time PCR or real-time RT-PCR with a TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA), as previously described.[1)]

Among all of the methods examined in this study, the visual colorimetry method was the easiest to use, and was not affected by DNA, RNA, or protein, but it was less sensitive to humic acids than the others (Fig. 1C and Table 1). In addition, this method determined a rough quantity rather than a precise quantity of humic acids. Thus, it is useful only when high levels of humic acids must be determined roughly, for example, in evaluation of it in an early step of DNA/RNA extraction from soil.

All of the visible and ultraviolet spectroscopic methods showed similar sensitivity to humic acids and similar linear ranges of detection (Table 1). These methods were not affected by DNA or RNA, and were affected by protein only when the concentration of protein was very high. Since such a high concentration of protein normally does not present after phenol extraction followed by spin column purification during DNA/RNA extraction, even if an extraction buffer containing skim milk is used, as reported previously,[7,8)]

[†] To whom correspondence should be addressed. Tel: +81-29-838-8262; Fax: +81-29-838-8199; E-mail: ftakeshi@affrc.go.jp
Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction

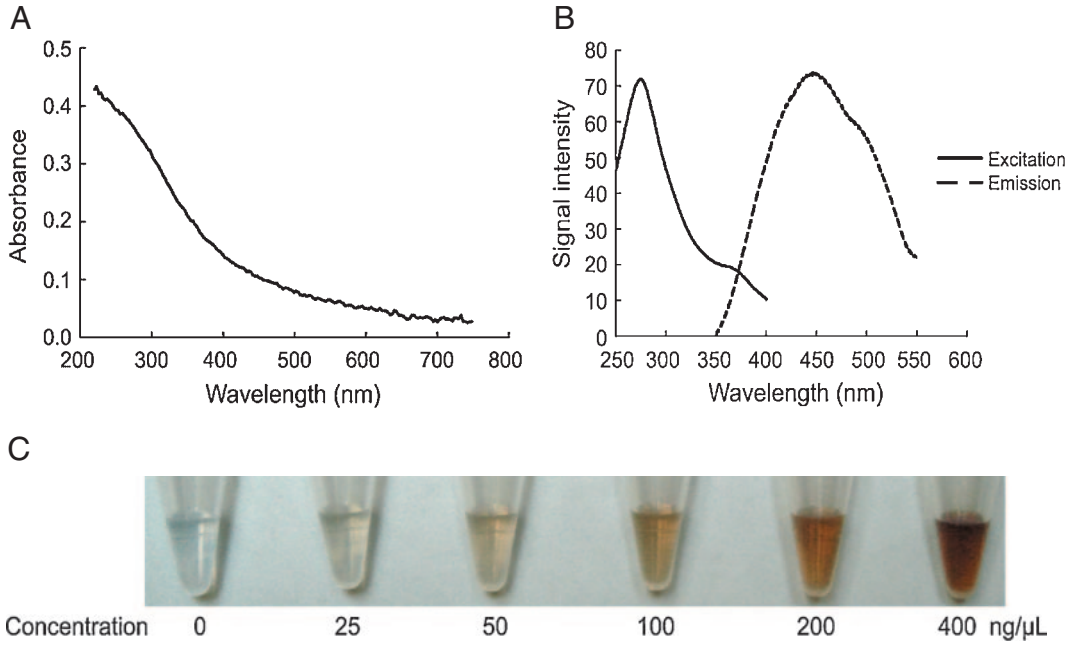

Fig. 1. Ultraviolet-Visible Absorption Spectrum (A), Fluorescence Spectrum (B), and a Set of Serial Diluted Solution (C) of a Commercial Humic Acid.

Table 1. Comparison of Methods of Measuring Humic Acids

Methods	Detection limit (ng/μL)	Linear range (ng/μL)	Linearity R^2	Disturbance[a]			
				RNA (ng/μL)	DNA (ng/μL)	BSA[b] (ng/μL)	Skim milk[b] (ng/μL)
Visual colorimetry[1)]	25	—	—	No effect	No effect	No effect	No effect
A_{465}[5)]	—	5–500	0.9996	No effect	No effect	>1,000	>1,000
A_{320}[3)]	—	5–200	0.9993	No effect	No effect	>500	>500
A_{340}[4)]	—	5–500	0.9998	No effect	No effect	>1,000	>1,000
A_{350}[2)]	—	5–500	0.9997	No effect	No effect	>1,000	>1,000
$\lambda_{excitation}/\lambda_{emission}$							
276/445[1)]	—	0.05–5	0.9997	>50	>20	>5	>1
471/529[4,6)]	—	0.05–20	0.9995	>100	>10	>50	>100

[a]Disturbing molecules affect the measurement of humic acids only when their concentrations are higher than the thresholds presented here.
[b]The disturbance of protein was examined using BSA (bovine serum albumin) and skim milk.

disturbance of protein is negligible in the determination of humic acids using visible or ultraviolet spectroscopic methods (data not shown).

The two fluorescence spectroscopic methods showed the highest sensitivity to humic acids among all of the methods we examined (Table 1). A previous report concluded that 10 ng/μL of DNA or 2 μg/μL of BSA did not affect the determination of humic acids at a high concentration (50 ng/μL).[4)] Since a high concentration of humic acids can easily be determined by visible and ultraviolet spectroscopic methods, we tested disturbance of DNA, RNA, and protein on the determination of low-level humic acids, and found that DNA, RNA, and protein do affect the determination (Table 1). This suggests that proper dilution of samples might be required to avoid disturbance by DNA, RNA, or protein when fluorescence spectroscopy is used.

To determine under what conditions the aforementioned methods are suitable to measure co-extracted humic acids in the extracted nucleic acids, we collected information from papers published previously. In experiments not sensitive to humic acids, such as DNase I or RNase digestion, transformation and nucleic acid hybridization, in which humic acids at lower than 100 ng/μL do not have a strong effect on experiments,[9,10)] even the visual colorimetry method is sufficient. However, in experiments sensitive to humic acids, such as restriction enzyme digestion, in which several ng per μL of humic acids inhibit enzyme activity significantly,[9)] fluorescence spectroscopy might be more helpful to measure low-level humic acids precisely if disturbance by DNA can be avoided. For PCR or real-time RT-PCR, however, there were no clear data showing the effects of humic acids on these reactions. Hence we evaluated the effects of humic acids on them (Fig. 2). Although we found information on the effects of humic acids on PCR,[9,11)] the lowest level of humic acids that affected PCR significantly was ambiguous because the values in the two reports were different from each other. To clarify this ambiguity, we conducted real-time PCR by the addition of different quantities of humic acids to the reaction mixtures. As shown in Fig. 2A, determination of abundance of genomic DNA was significantly affected by humic acids at a level of ≥10 ng/μL, which was consistent with one of the reports,[11)] suggesting that this value is reliable. Since

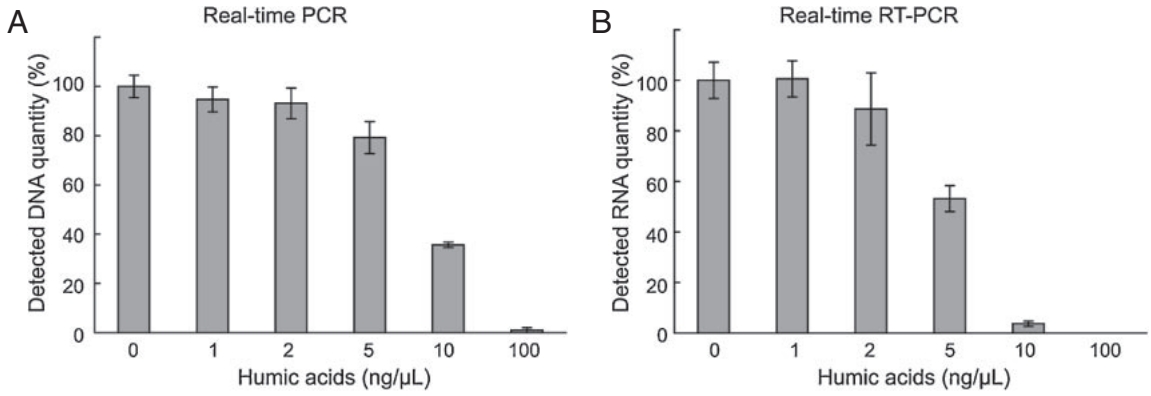

Fig. 2. Inhibitory Effects of Humic Acids on Real-Time PCR (A) and Real-Time RT-PCR (B).
Two μL of DNA or RNA was used in each 50-μL reaction mixture, and triplicate samples were examined. Error bars indicate standard derivations.

we did not find any information on the effects of humic acids on RT-PCR, one of the popular techniques in molecular biology laboratories, we conducted real-time RT-PCR. As shown in Fig. 2B, determination of abundance of RNA was affected by humic acids at a level of $\geq$5 ng/μL. This suggests that RT-PCR is more sensitive to humic acids than PCR, probably because disturbance of humic acids occurred in two reactions, both the reverse transcription and the PCR. Since the methods of A_{465}, A_{320}, A_{340}, and A_{350} detected humic acids at levels as low as 5 ng/μL, all of the visual and ultraviolet spectroscopic methods were sufficient to evaluate the quality of soil DNA and RNA for routine PCR or RT-PCR analysis.

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