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Novel and Rapid *on site* Nucleic Acid Quantification Platform Customised for Archaeological Science

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Abstract

Recent advances in DNA-related technologies have much facilitated bioorganisms identifications relying on the DNA sequences from archaeological and environmental samples including sediments/soils and water. Efficient DNA extraction methods are being thereby in pursuit and the efficacies of each extraction method have been long discussed without understanding the "initial" amount of DNA originally contained in the samples. Knowing the initial DNA concentration would feasibly allow sample selections at the archaeological excavation sites. We herein introduce a novel method substantially realising extraction-free quantification at the excavation site not only of double-stranded DNAs (dsDNAs) but also single-stranded DNAs (ssDNAs) and mirco RNAs (miRNAs) by commercially-available mobile fluorometer independent of any contaminants, contributing to the instant sample selections. Furthermore, we show that nucleic acid contents (dsDNAs, ssDNAs and miRNAs) stayed almost the same from surface to bottom (loam) layers (~1,500 ng (dsDNA), ~5,000 ng (ssDNA), ~4,000 ng (miRNA) per mL of the 20% (w/v) soil solutions), possibly implying that nucleic acids might have migrated vertically between strata during a long period via natural phenomena such as rainfall permeation into the soil. This extraction-free quantification method is notably characteristic in its rapidity, simplicity as well as accuracy, hopefully paving the way for future advances in archaeological science and its related area.

Keywords: *on site* nucleic acid quantification; Mobile fluorescent quantification

Abbreviations:

dsDNA: double-stranded DNA; ssDNA: single-stranded DNA; miRNA: micro RNA, eDNA: extracellular DNA; iDNA; intracellular DNA; UV: Ultra Violet; NGS: next generation sequencing.

Introduction

Recent advances in molecular biology have greatly contributed to archaeological research, thus propagating drastic changes in archaeological methodology. Especially, developments in nucleic acid sequence analysis together with resulting genome database have much facilitated archaeological identification of bioorganisms, for example, originated from bones [1-2], sediments [3-4], pollen [5], charred grains [6], and parasite eggs [7]. However, from archaeological points of view, experimentally-accessible samples suitable for further sequential analysis are typically very limited, mainly depending upon either the storage conditions of archaeological samples or the nucleic acid extraction methods [8-9]. Extraction methods suitable for archaeological samples have been thus extensively explored [10-11].

In the case of excavations outside of the native country, it would be desirable to select at the excavation sites which environmental samples (e.g. soils, sediments and water) should be transferred to the laboratory, which may cause some difficulties between nations. Therefore, rapid, simple and cost-effective methodologies for achieving nucleic acid quantification *on site* (i.e. at the excavation sites) have long been considered ideal in archaeological science.

Various approaches are archaeologically attempted for establishing nucleic acid extraction methods from cultural properties. For example, in order to initially elute nucleic acids from soils or sediments, phosphate buffer-based procedures have been broadly recognised [11-14]. Although there are examples which report successful nucleic acid extractions from soil samples using phosphate buffer-based methods evaluated from the quantifications "after" the purification, however, it is generally not straightforward to determine initial amount of the nucleic acids originally included in the samples. It should be also taken into account that, in the case of quantifying environmental DNA, the total DNA obtained from soil ought to be regarded as the sum of extracellular DNA (eDNA) and intracellular DNA (iDNA). The term, eDNA, stands for the DNA released out of bioorganisms into the environment which might have existed in soil by robust binding to minerals, being ubiquitously excreted from living microorganisms followed by binding cohesively to soils or sediments [12], whilst iDNA refers to the DNA engulfed by membranes (or capsules) of living bioorganisms, such as virus, bacteria and fungi. Separation methods between eDNA and iDNA have thus been developed [13-14]. Since eDNA is usually a target in the research areas of archaeology and environmental science, effective elimination or inactivation of peripheral nucleases that might digest eDNA is also necessary.

In laboratories, nucleic acid quantifications are generally performed by using spectrophotometers, measuring the maximum absorption at the wavelength of 260 nm. In terms of environmental samples including soils and water, however, due to the contaminants which can hinder the spectrophotometric measurements in the UV region are inclusively contaminated (e.g. proteins and other chemical compounds which characteristically absorbs lights with wavelengths around UV region), spectrophotometric quantification is practically impossible without the robust extraction and the following purification procedures. The extraction from soils appears to be generally accomplished by utilising commercially available extraction/purification kit including beads-based DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germany) not only in archaeological research [15-16], but also in environmental research [17-18] and forensic sciences [19]. To assess the DNA concentration of crude samples, fluorometric quantification methods including the use of Hoechst 33258 and 4'-6-diamidino-2-phenylindole (DAPI) would be applied [20]. However, these fluorescent dyes are able to specifically bind to double-stranded DNAs (dsDNAs) [21-23], and therefore cannot be used for quantifying single-stranded DNAs (ssDNAs) or RNAs.

We herein report a novel platform which substantially allows rapid, simple and cost-effective nucleic acids (dsDNAs, ssDNAs and microRNAs (miRNAs)) quantification at the excavation sites. We show that quantifications of the nucleic acids independent of either extraction procedures or any contaminants can be achieved by utilising handy fluorometer, whose fluorescent dyes are able to specifically recognise dsDNA, ssDNA or miRNA respectively. This fluorescence-based nucleic acid quantification, typically used for specific purposes including next generation sequencing (NGS) [24], is more precise and sensitive than the conventional spectrophotometric method, desirably allowing for specific and accurate quantifications of both DNAs and RNAs even though samples are environmentally adulterated. Moreover, since these fluorometers are generally portable, nucleic acid quantifications at excavation sites can be easily achieved if only electric supply is stable. In addition, we also report that nucleic acids are found to be of much abundance even in Kanto loam layer which had been formed by ancient volcanic activity of Mt. Fuji (Japan) in the Quaternary period, raising the possibility that nucleic acids can be vertically mobile through natural phenomena such as rainwater permeation. This supposition further raises a concern that the nucleic acids excavated from a certain geographical stratum might have been descendent from those of different eras.

Materials and Methods

Soil Sampling and *on site* Nucleic Acid Quantification

Soil samples herein were from the Nakayamakokunai archaeological site in Hokuto city, Yamanashi prefecture, Japan, where numerous pit dwellings belonging to the 10th - 11th century AD were excavated [25]. The soil samples were carefully harvested with clean spatula and collected into sterilised tubes at 30 cm intervals from the surface layer (reddish clay) to Kanto loam layer (5 points; exemplified in Figure 1(a)~(c)).

Nucleic acid quantifications herein were carried out by Qubit 4 Fluorometer (Thermofisher Scientific, MA; Figure 1(d)). For quantifications of dsDNAs, ssDNAs and miRNAs, Qubit™ dsDNA HS Assay Kit (#32851, Thermofisher Scientific), Qubit™ ssDNA Assay Kit (#Q10212, Thermofisher Scientific) and Qubit™ microRNA Assay Kit (#Q32880, Thermofisher Scientific) were employed respectively. These QubitTM assays appears to be developed from the original sets of reagents called Quant-iTTM assays comprising PicoGreenTM (dsDNA), OliGreenTM (ssDNA), and RiboGreenTM (RNAs) [26]. Each fluorescent dye is known to specifically bind to each substrate proportionally, which give rise to respective characteristic fluorescence, generally perceived to be more accurate than spectrophotometric quantification [27]. For successful nucleic acid quantification at the excavation site, stable electric supply is to be required for the fluorometer. Thus an electric dynamo was prepared ahead of the quantifications. For quantification, 0.1 g of each soil samples were soaked in autoclaved 0.5% (w/v) sodium phosphate solution (~pH 12.5) to prepare 20 % (w/v) soil solution (0.1 g soil in 0.5 mL solution). 20 % (w/v) soil solution were then vigorously shaken, allowed to sediment for 30 minutes, and the aliquots of the supernatant were used for the quantifications. The quantification method was in accordance with the manufacturer's manual, with 10

µL of the analyte sample plus 190 µL of quantification reagent.

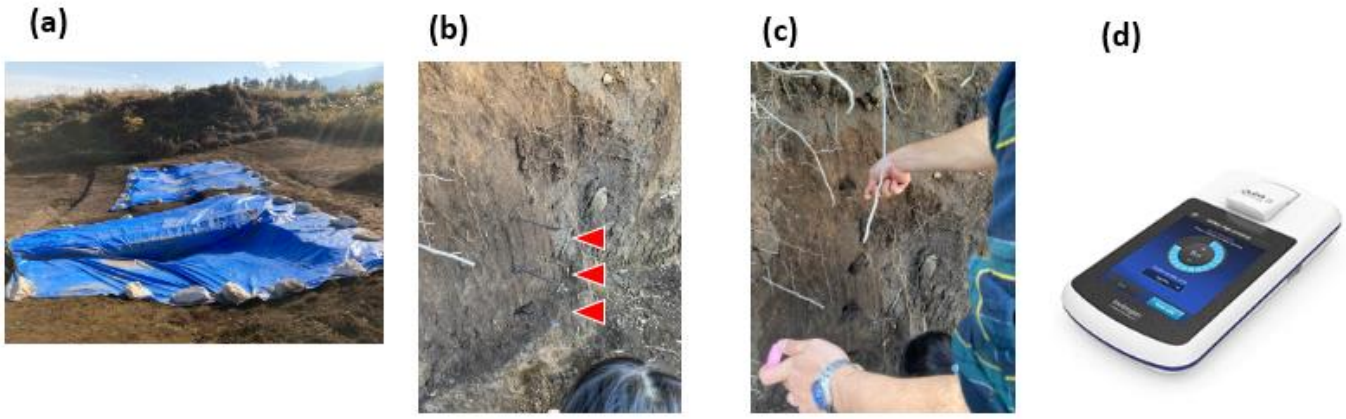

Figure 1: Soil sampling at the excavation site at Hokuto city, Yamanashi, Japan. (a) Landscape of the excavation site. (b) (c) Soil sampling alongside the strata from the surface to Kanto loam layer by 30 cm interval each (surface, upper, middle, lower and the loam layer). The red wedges in (b) represents spatulas inserted into each layer as markers for sampling. (d) Qubit4 fluorometer (Thermofisher Scientific; with a size of 13.6 cm × 25 cm × 5.5 cm) used in this study.

Condition for the Elution of Nucleic Acids from Soils

Nucleic acids can be easily eluted from the soil samples at alkaline conditions [11-14], yet nucleic acids are prone to hydrolysis upon alkaline treatment, thus to be adversely damaged [27]. Therefore, it is assumed to be critically important to carefully determine the optimum elution condition (e.g. types of buffer, pH, temperature and time for elution). For exploring the optimum condition, the buffer and the temperature conditions were fixed to phosphate buffer and room temperature respectively as these conditions have widely been used in the archaeological researches [11-14]. Additionally, duration for elution was set to be 30 minutes in this study since minutes-order elution has generally been employed in archaeological research [13-14], and also beneficial to practicing *on site* quantification method. Thus, in this study, pH was arranged to be the variables, and evaluated by the extraction yield (ng nucleic acid per mL elution buffer containing eluted nucleic acids) based on the fluorometric quantifications as described above. In search for the optimum pH, 0.5% (w/v) NaH_2PO_4, 0.5% (w/v) Na_2HPO_4 and 0.5% (w/v) Na_3PO4 solutions were thoroughly mixed so as to be adjusted to each desired pH ranging from pH 7.5 to 12.5 (pKa_2 = 7.21, pKa_3 = 12.67). Each quantification was performed three times (n=3).

Quantifications seeking for optimum pH condition were performed in the laboratory. The 20% (w/v) soil solutions at respective pH (pH 7.5 to 12.5) were thoroughly shaken and stood for 30 minutes at ambient condition, followed by centrifugation at 15,000 g for 15 minutes at room temperature. Each supernatant was cautiously pipetted into new tubes and subjected to the Qubit 4 Fluorometer quantifications. The soil samples were carefully handled so as to avoid contaminations from the experiment practitioners, e.g. appropriately using disposable gloves and clean labwares.

Results and Discussion

on site Nucleic Acid Quantification

Soil samples from the excavation site described above was subsequently subjected to Qubit 4 Fluorometer quantification, confirming that dsDNAs, ssDNAs and miRNAs *on site* quantifications were substantially feasible (Figure 1). We believe that this approach serves as an extraction-free nucleic acids quantification method with unprecedented rapidity and accuracy that can be applied at any excavation sites. Whereas the electricity for the fluorometer was supplied by an electric dynamo driven by petrol in this study, other types of electricity supplying devices such as portable batteries would also be well used for this purpose, facilitating *on site* quantifications to which this study pertains.

pH-Dependency of Nucleic Acid Elution from Soil Samples

Since the pH condition suitable for eluting nucleic acids from the sample soils appear to greatly vary according to literatures ranging from quasi-neutral [12] pH to 12.5 (i.e. Na_3PO4 solution) [13-14], the optimum pH was re-examined in this study, showing that dsDNAs originated were best eluted at pH 12.5 from any layers (Figure 2).

Elution of dsDNAs, ssDNAs and miRNAs from the Each Layer

Qubit 4 fluorometer has a feature that the concentrations of dsDNAs, ssDNAs and miRNAs can be separately and specifically assessed. Taking advantage of this

feature, we examined the dsDNAs, ssDNAs and miRNAs concentrations. When eluted at pH 12.5 from the each layer, ssDNAs and miRNAs are considerably abundant in any layers examined (Figure 3), yielding about twice to three times as much as dsDNAs in mass, raising the possibility that dsDNAs from ancient living organisms might have digested by the exogenous DNases in the soil mostly produced by bacteria and fungi, thus giving rise to these abundancies. For the miRNAs, since Qubit™ microRNA Assay Kit is capable of quantifying not only miRNA but also short RNAs according to the manufacturer's instruction, the miRNAs quantified herein may comprise miRNAs as well as short, fragmented RNAs possibly derived from degraded RNAs of dead bioorganisms.

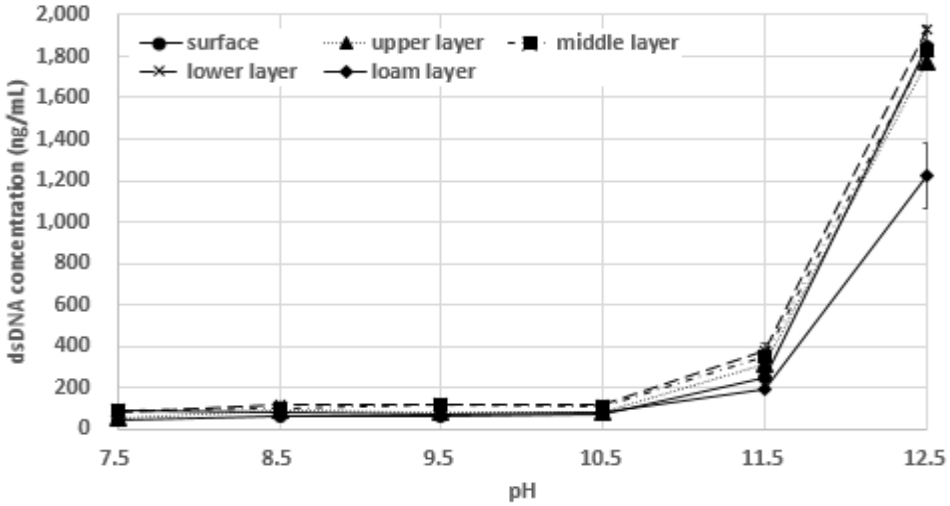

Figure 2: pH dependency of dsDNA elution from the soil samples, quantified by Qubit™ dsDNA HS Assay Kit as described above, showing efficient elution of dsDNA at alkaline condition (~pH 12.5). Ahead of dsDNA, ssDNA and miRNA quantifications, negative controls were examined respectively using 0.5% (w/v) phosphate buffer at each pH. Qubit4 fluorometer showed "out of detectable range", confirming no signs of nucleic acids in the negative controls.

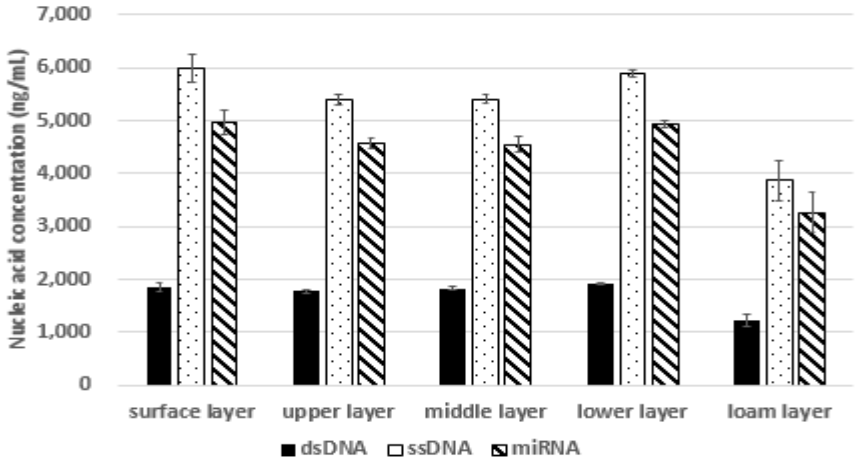

Figure 3: Respective quantifications of dsDNA, ssDNA, miRNA at each stratum, showing that all kinds of nucleic acids are abundant even in the deep stratum.

Additionally, it is noteworthy that rigid dsDNA elution was confirmed even in Kanto loam layer, formed by Mt. Fuji's volcanism during Quaternary period, as well as in other layers. Since the numbers of bacteria and fungi decrease in

proportion to the depth [28], we originally expected less nucleic acids content in the Kanto loam layer. However, this study clearly demonstrated that the dsDNAs (as well as ssDNAs and miRNAs as illustrated in Figure 3) eluted from the volcanic soil were comparable to those from other layers, raising the possibility that nucleic acids are able to vertically migrate one another. This possibility further implies that the age estimate depending upon the nucleic acids (e.g. ^{14}C dating) from one layer might lead to erroneous age assumption because of this vertical migration and/or the living microbe contamination.

Conclusion

We herein exemplified the versatility of extraction-free nucleic acid quantification, allowing for the practical measurements at the excavation sites by means of the commercially available mobile fluorometer. Whilst various extraction methods for eluting environmental DNAs have been exploited to date, to our knowledge, none of those methods was aimed for quantifying the original mass of DNAs. This extraction-free nucleic acid quantification method, in contrast, is likely to be a platform that facilitates further improvements toward the accurate estimation of the concentrations of nucleic acids despite the soil crudity, hopefully contributing to the further exploits in environmental DNA extraction methods. In this connection, as the total DNAs according to this study should be regarded as the sum of iDNAs and eDNAs, filtration procedures for separating between iDNAs and eDNAs prior to the quantifications may be required depending upon the research purpose.

In archaeological research, since transporting the soil samples to other countries (or researchers' own countries) might cause diplomatic complications, it is generally favoured that the soil samples must be minimised in weight, as well as in volume. The application of the quantification method exemplified herein may help such transportation problem by ensuring the sample selections at the excavation sites.

Through the application of extraction-free nucleic acid quantification method, a considerable amount of nucleic acids was found to definitely exist even in the volcanic layer, raising the possibility that nucleic acids could be vertically mobile between layers. Therefore, future development of reliable dating methodologies for nucleic acids independent of those estimated from the stratum is awaited. Furthermore, according to this study (Figure 3), as not only dsDNAs but also ssDNAs and miRNAs are abundantly present in every layer, we believe that methods for identifying bioorganisms relying on those ssDNAs and miRNAs (or short RNAs), for example, enzymatic cloning or amplification procedures specifically utilising soil ssDNAs or miRNAs as templates, would shed more light on the ancient biota in detail. It should be also noted that the yields of dsDNAs, ssDNAs and miRNAs may well be variable between geological strata because the absorptions of nucleic acids by soils are generally known to greatly vary dependent upon the soils' composition, shape, particle size, and porosity [29]. For instance, Ogram et al. analytically showed that S_{max} (maximal DNA binding (g) per unit soil sample (g)) of montmorillonite is 15.4 μg/g through experimental plots of Langmuir absorption isothermal equation [12]. The method exemplified in the present study is capable of direct quantifications of nucleic acids, thereby facilitating experimental procedures pursuing for the absorption constants of each material towards nucleic acids.

Recent advances in DNA-related technologies have much promoted archaeological science and related area such as environmental and ecological research. For example, the use of nanopore sequencing coupled with DNA barcoding technique was reported [30], thereby enabling NGS-based analysis targeting on specific species of interest outside of laboratories. Although modern advances in DNA sequencing technologies have successfully allowed such "field laboratories", it should be taken into account, for instance: (1) that, when eluting nucleic acids from soil samples, longer exposure to alkaline solution should be avoided, which might result in hydrolytic degradation of nucleic acids [27]; (2) that DNA postmortem degradation (C-to-T and/or G-to-A change) might lead to erroneous interpretation as to the sequencing results [31-32]; and (3) that the extracted nucleic acids should be regarded as a mixture of both the intracellular nucleic acids from living cells and the extracellular (or environmental) nucleic acids without any special filtration procedures. In order to substantially separate between intra- and extracellular DNAs, Skoglunda et al. had developed, for example, data filtering methodology [31]. As the eluted nucleic acids (dsDNAs, ssDNAs and miRNAs) according to this study should also be regarded as the mixture of intra- and extra-ones, some "sieving" procedures either statistically or chemically would of course be required for the satisfying sequence results.

For sequence analysis of DNAs, ng to μg order DNAs with >100 bps are generally to be required. DNeasy PowerLyzer PowerSoil Kit (Qiagen), for example, can clean up dsDNAs with >100 bps from the soils of interest. With using this purification kit, we further attempted dsDNA purification from the soils of the five layers listed above, which gave rise to ~400 ng of purified dsDNA per 1 g of each soil. Although ~7,500 ng dsDNA per 1 g soil (i.e. ~1,500 ng per mL of the 20% (w/v) soil solutions) were found to initially exist according to the Qubit4 quantifications (Figure 3), we suppose that this great difference between the crude and purified dsDNA amounts could attributed to the distribution dependent on the dsDNA's length. As PicogreenTM dye can recognize 4 bps of dsDNA [33], this Qubit4 quantification result in principle could be dsDNA concentrations with >4 bps. We thus imagine that DNA purification platform targeted on shorter DNAs (<100 bps) would give rise to more genetic information on the biota of interest.

This study thus illustrated the potential versatility of the fluorescent quantification method characterised by its simplicity, mobility and rapidity, allowing for "*on site*" (at the excavation sites) selections which sample should be carried back to the laboratories. We expect that this *on site* nucleic acid quantification method could contribute to future advances in archaeological science by promoting DNA-based bioorganisms identification technologies.

Competing Interests

The authors declare no competing interests.

Author Contribution

H.O., M.U., A.F., K.Y. set forward the research plans, H.O., M.H., T.K.U. did the experiments, and H.O., T.K.U., Y.K., K.A., R.F., K.M., M.U., A.F., K.Y., T.I. prepared the manuscript.

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