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Formic acid hydrolysis/liquid chromatography isotope dilution mass spectrometry: An accurate method for large DNA quantification

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ABSTRACT

Liquid chromatography-isotope dilution mass spectrometry (LC-IDMS) with formic acid hydrolysis was established for the accurate quantification of λDNA. The over-decomposition of nucleobases in formic acid hydrolysis was restricted by optimizing the reaction temperature and the reaction time, and accurately corrected by using deoxynucleotides (dNMPs) and isotope-labeled dNMPs as the calibrator and the internal standard, respectively. The present method could quantify λDNA with an expanded uncertainty of 4.6% using 10 fmol of λDNA. The analytical results obtained with the present method were validated by comparing with the results of phosphate-base quantification by inductively coupled plasma-mass spectrometry (ICP-MS). The results showed good agreement with each other. We conclude that the formic acid hydrolysis/LC-IDMS method can quantify λDNA accurately and is promising as the primary method for the certification of DNA as reference material.

1. Introduction

DNA quantification is carried out in many areas of specialty, including food analysis, medicine, and pharmacy. In food analysis, genetically modified organism (GMO) or microbial contamination of food is quantified by DNA measurement. Because the detection of GMO contamination is important to meet legislative requirements by country [1], a method for the accurate quantification of GMOs is needed. The detection of disease-causing microorganisms in medicine or the quality control of nucleic acid medicines in pharmacy is carried out as routine work by measuring DNA. DNA size of interest ranges from a few nucleotide base pairs (bp) as seen in single-stranded oligonucleotides to many millions of bp typical of double-stranded eukaryotic genomes [2]. Routine DNA measurement could benefit greatly from more accurate and reliable DNA measurement; however, neither an appropriate DNA reference material nor a suitable method for DNA quantitative measurement is available.

For the determination of DNA concentration, ultraviolet (UV) absorbance and fluorescence measurements are generally conducted [3,4]. The measurement of UV absorbance at 260 nm, which is the local absorption maximum of DNA, is commonly carried out for the determination of DNA concentration; however, the conversion factors for this method are not metrologically traceable. Other popular methods include the PicoGreen assay and the real-time quantitative polymerase chain reaction (qPCR) in combination with a fluorescent dye [4,5]. However, because of problems arising from the use of conversion factors in UV absorbance measurement and the dependence of the PicoGreen assay and qPCR on external calibrators, these methods fail to accurately quantify DNA [6]. Therefore, an accurate and traceable method for the characterization of DNA certified reference material (CRM) for effective comparison of quantification, quality control in routine work, and method validation is required.

Five methods that have potential metrological traceability include direct counting [7], digital PCR [8], quantitative nuclear magnetic resonance (qNMR) [9], inductively coupled plasma-optical emission spectrometry (ICP-OES) [3,10,11], and liquid chromatography-isotope dilution mass spectrometry (LC-IDMS) [6,12–15]. The direct counting method and digital PCR address different measurands, such as countable particle and amplifiable targets, respectively, rather than total DNA. qNMR lacks sufficient sensitivity for total DNA measurement. ICP-OES and LC-IDMS are the methods of choice for the accurate quantification of total DNA. ICP-OES is based on the measurement of DNA phosphorus (P) content. LC-IDMS, on the other hand, measures DNA compounds, such as deoxynucleotides (dNMPs) or deoxynucleosides (dNs), after the enzymatic digestion of DNA [6,12–16]. Because LC-IDMS can quan-

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tify DNA indirectly through the quantification of dNMP or dNs, a DNA standard having an accurate value, such as concentration or mass fraction, is not required. Furthermore, LC-IDMS is fully traceable to International System (SI) units by using an appropriate single-nucleotide standard.

For the accurate quantification of DNA by LC-IDMS, complete digestion and restriction of the over-decomposition of DNA compounds are required. As regards DNA digestion, two methods have been reported. One is enzymatic digestion, which has already been mentioned earlier, and the other is acid hydrolysis. The former can restrict the over-decomposition of dNMPs or dNs by optimizing the reaction conditions. However, large DNAs having more than 10,000 bp might not be digested completely, as was shown by Dong et al. [6], who applied enzymatic digestion to λDNA quantification but had to rely on ultrasonication with a special instrument prior to the enzymatic digestion. Acid hydrolysis could hydrolyze DNA into nucleobases completely, but the nucleobases were over-decomposed by hydrochloric acid or formic acid [2,17–20]. In hydrochloric acid hydrolysis, accurate measurement was difficult due to the influence of over-decomposed compounds. In formic acid hydrolysis, cytosine was deaminated to uracil [2]. However, if the over-decomposition of nucleobases could be well controlled, acid hydrolysis would be a promising tool for the digestion of DNA, particularly large DNA. Furthermore, uncertainty arising from the acid hydrolysis of DNA would be reduced because the DNA digestion step would be less than the enzymatic quantification with ultrasonication.

In this study, we developed a method for the accurate quantification of large DNA, which involves acid hydrolysis with LC-IDMS. For DNA digestion, formic acid was chosen because the reaction of formic acid was milder than that of hydrochloric acid. As sample, we used λDNA, which is the genomic DNA of bacteriophage lambda, and dsDNA of 48,502 bp. By using dNMPs or isotope-labeled dNMPs (LdNMPs) as the calibrator or the internal standard, hydrolyzing dNMPs and LdNMPs to nucleobases, and optimizing the reaction conditions, the accurate quantification of nucleobases was achieved. We quantified P in the same λDNA sample by ICP-mass spectrometry (ICP-MS) and compared the results of the developed method with the results of ICP-MS.

2. Experimental

Water used for preparation and dilution was purified water from Milli-Q Advantage (Millipore, Tokyo, Japan).

2.1. λDNA solution

λDNA solution was purchased from Nippon Gene (Tokyo, Japan). The amount of substance content of λDNA solution was roughly estimated to be 22 pmol/g with a UV spectrometer (BioSpec-nano, Shimadzu). λDNA quality was evaluated by gel electrophoresis using 0.3% agarose H (Wako, Osaka, Japan). Because a single band measuring approximately 50,000 bp was observed, the λDNA was considered intact. λDNA solution was diluted to 0.33 pmol/g with water and used as sample.

2.2. Reagents and samples

2′-Deoxyadenosine 5′-monophosphate disodium salt (dAMP), 2′-deoxycytidine 5′-monophosphate disodium salt (dCMP), 2′-deoxyguanosine 5′-monophosphate disodium salt (dGMP), and 2′-deoxythymidine 5′-monophosphate disodium salt (dTMP) were purchased from MP Biomedicals (CA, USA). ^{13}C- and ^{15}N-labeled dAMP, dCMP, and dTMP were purchased from Spectra Stable Isotopes (MD, USA), and ^{13}C- and ^{15}N-labeled dGMP was purchased from ISOTEC International (GA, USA). LC/MS grade acetic acid, LC/MS grade methanol, special grade formic acid and 3-(Trimethylsilyl)1-propanesulfonic acid-d_6 sodium salt (DSS-d_6) reference material was purchased from Wako. Potassium hydrogen phthalate (PHP, NMIJ CRM 3001-b) was from the National Metrology Institute of Japan (NMIJ, Ibaraki, Japan). Ultrapure grade nitric acid, phosphate ion standard solution (JCSS), and cobalt (Co) standard solution (JCSS) were purchased from Kanto Chemical (Tokyo, Japan).

Table 1
The m/z and collision energy of nucleobases and labeled nucleobases by LC/MS/MS.

Analyte	m/z	Collision energy (eV)
A	136.00 –> 119.15	−28
^{13}C- and ^{15}N-labbeled A	146.0 –> 128.0	
C	112.0 –> 40.0	−37
^{13}C- and ^{15}N-labbeled C	110.0 –> 54.05	
G	152.0 –> 110.0	−22
^{13}C- and ^{15}N-labbeled G	162.0 –> 117.05	
T	127.00 –> 54.05	−27
^{13}C- and ^{15}N-labbeled T	134.0 –> 58.0	

2.3. Equipment and measurement

2.3.1. LC/MS/MS

For nucleobase measurement, an LC-10A series HPLC (Shimadzu, Kyoto, Japan) and an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu) were used. Nucleobase separation was achieved with Kinetex XB-C18, 4.6 mm × 250 mm, 5 μm (Phenomenex, CA, USA) at the flow rate 0.5 mL/min. The mobile phase used for nucleobase separation was 8% methanol with 0.1% acetic acid in water. MS conditions were electrospray ionization (ESI) and multiple reaction monitoring (MRM) in the positive ion mode. Flow rate of nebulizing gas was 3 L/min, desolvation line (DL) temperature was 250 °C, heat block temperature was 400 °C, and flow rate of drying gas was 15 L/min. Ions monitored for Adenine (A), Cytosine (C), Guanine (G), Thymine (T), ^{13}C- and ^{15}N-labeled A, ^{13}C- and ^{15}N-labeled C, ^{13}C- and ^{15}N-labeled G, and ^{13}C- and ^{15}N-labeled T, and collision energy are shown in Table 1.

2.3.2. ICP-MS

For P measurement, ICP-MS (ELEMENT XR, Thermo Fisher Scientific, Tokyo, Japan) was used. Incident Rf power was 1.5 kW and reflected power was <1 W. The flow rates of argon as outer gas, intermediate gas, carrier gas, and make-up gas were 15 L/min, 0.8 L/min, 1.0 L/min, and 0.2 L/min, respectively.

2.3.3. NMR

All ^{1}H NMR spectra were obtained with a VNS 600 spectrometer (Agilent Technologies, California, USA) operating at 599.90 MHz with ^{1}H resonance frequency. A typical set of ^{1}H NMR experimental parameters was as follows: 59523.8 Hz (99.2 ppm) spectral width, 4.0 s acquisition time, 13.0 μs (90°) pulse width, 60 s relaxation delay, and 32 transients acquired. Data processing was performed using MestReNova ver.9.0.1. All signals were integrated without including the ^{13}C satellite signals [21].

2.4. DNA quantification by acid hydrolysis

2.4.1. Amount of substance content measurement of each dNMP standard solution

Each dNMP standard solution was prepared by dissolving in water and the amount of substance content of each dNMP standard solution was determined by qNMR. Briefly, the solvent of weighed dNMP standard solution was removed by evaporation