Quantification of Cell-Free DNA: A Comparative Study of Three Different Methods

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pISSN: 2384-2458
eISSN: 2288-7261

Background: Cell-free DNA (cfDNA) provides unique potential as a biomarker for cancer patients or in the field of prenatal care. Following extraction from plasma, cfDNA should be quantified before downstream analysis. The standardization of the quantification methods is essential for application of these techniques to laboratory practice. We aimed to compare three different quantification methods (spectrophotometry, fluorometry, and the electrophoresis-based method) and to determine the contributor(s) to the differences, if any.

Methods: A total of 135 plasma samples obtained from cancer patients (n=71) and normal individuals (n=15) and cfDNA were extracted from the samples. The extracted cfDNA was quantified using three different methods: NanoDrop spectrophotometry, Quantus fluorometry, and the electrophoresis-based 4200 TapeStation method.

Results: NanoDrop exhibited the highest estimates (median, 12.3 ng/uL). The estimates of Quantus (median, 0.33 ng/uL) were higher than those of 4200 TapeStation (median, 0.22 ng/uL). Quantus and TapeStation were significantly correlated ($r=0.802$, $P<0.001$). The Bland-Altman plot showed a positive bias of 0.24 ng/uL for Quantus compared with TapeStation. The difference between Quantus and TapeStation values demonstrated a significantly moderate correlation with the amount of high molecular weight DNA ($r=0.357$, $P<0.001$).

Conclusions: Nanodrop is considered as a non-specific method of cfDNA quantification. Quantus depicted more specific quantification compared to Nanodrop; however, TapeStation may be used in parallel or as a complementary method as it enables to determine the size distribution of DNA fragments.

(Lab Med Qual Assur 2019;41:214-219)

Key Words: Cell-free nucleic acids, Spectrophotometry, Fluorometry

Received July 2, 2019, **Revision received** August 8, 2019, **Accepted** August 22, 2019

INTRODUCTION

Cell-free DNA (cfDNA) is a subset of circulating extra-cellular DNA in plasma, which is released from cells mostly through apoptosis, necrosis, and active secretion [1]. The cfDNA is easily obtained by using minimally invasive procedures and has unique potential biomarkers for non-invasive prenatal testing and cancer management [2,3]. The recent advances in molecular technologies such as next-generation sequencing (NGS) and digital polymerase chain reaction (dPCR) have enabled routine analysis in clinical laboratories [4].

The standardization of the analytical conditions to

detect the genetic alterations in cfDNA still remains a major challenge [5]. After isolation from plasma, cfDNA is quantified before downstream analysis (e.g., NGS and dPCR). An accurate measurement of cfDNA, as a stringent quality control of cfDNA isolation, is a critical preliminary step for these downstream analyses [6]. Several methods have been described to quantify cfDNA. Quantitative PCR (qPCR) has been used to measure DNA concentration by targeting various pre-selected DNA sequences, and recently more rapid and cost-effective methods such as spectrophotometry, fluorometry, and electrophoresis-based methods are being used [5]. Spectrophotometers (e.g., NanoDrop) measure the

maximal absorbance at 260 nm. Fluorometers (e.g., Qubit and Quantus) use fluorescent probes which determine the DNA concentration. Each fluorescent probe is specifically bound to one type of molecule (double-stranded DNA [dsDNA] or single-stranded DNA [ssDNA]), which enables us to measure relatively low concentrations of cfDNA [7]. Electrophoresis-based instruments (e.g., TapeStation and Bioanalyzer) allow fluorometric quantification and visualize the concentration of cfDNA by size. However, there is lack of published evidence to characterize the differences between these methods.

The purpose of this study was to perform a head-to-head comparison among the aforementioned three different quantification methods (spectrophotometry, fluorometry, and electrophoresis-based methods) and to determine the contributor to the any differences between them.

MATERIALS AND METHODS

1. Blood collection

A total of 135 blood samples from 71 patients (54 with colorectal cancer and 17 with pancreatic adenocarcinoma) and 15 normal individuals were collected, including 29 patients from whom multiple sequential blood samples were taken during follow-up. Blood samples (3.0 mL) were collected into BD Vacutainer K₂ EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After the drawing of blood, the tubes were further processed within 3 hours.

2. Plasma preparation

Blood samples were centrifuged at $1,600\times g$ for 10 minutes, at room temperature. A total of 1.5 mL of each plasma sample was transferred to 1.5 mL microtubes (Eppendorf, Hamburg, Germany). In order to remove any residual blood compartments, the plasma fractions were sequentially centrifuged at $13,000\times g$ for 10 minutes. The supernatant was transferred to clean 1.5 mL microtubes and stored at -80°C until further analysis.

3. DNA isolation and quantification

cfDNA was manually extracted from plasma (1 mL)

using a QIAmp circulating nucleic acid kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The cfDNA was eluted in 50 uL Buffer AVE provided by Qiagen and stored at 4°C , until further analysis. The isolated cfDNA was quantified using three different methods: NanoDrop ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), Quantus fluorometer with the QuantiFluor ONE dsDNA kit for a specific quantification of dsDNA (Promega, Madison, WI, USA), and the microfluidic platform-based electrophoresis 4200 TapeStation instrument with high sensitivity D1000 Tape and reagents that enable the determination of the distribution of DNA fragments ranged between 50 and 1,000 bp (Agilent, Santa Clara, CA, USA). All the protocols followed the manufacturers' instructions.

4. Statistical analysis

The statistical differences in DNA concentration were tested using Friedman test, Wilcoxon signed rank test, and Mann-Whitney *U*-test. Pearson correlation and Bland-Altman plot were used to assess the correlation and differences between Quantus and TapeStation. *P*-values <0.05 were considered statistically significant.

RESULTS

The concentrations of isolated cfDNA were estimated using three different quantification methods (NanoDrop spectrophotometry, Quantus fluorometry, and the electrophoresis-based 4200 TapeStation method). The concentration estimates differed significantly among the different methods ($P<0.001$) (Fig. 1). The NanoDrop exhibited the highest estimates (median, 12.30 ng/uL; range, 9.5–23.9 ng/uL). The estimates of the Quantus fluorometer were higher than those of the 4200 TapeStation (Quantus: median, 0.33 ng/uL; range, 0.071–4.420 ng/uL; TapeStation: median, 0.22 ng/uL; range, 0.039–4.040 ng/uL).

The estimates of Quantus and TapeStation were significantly higher in cancer patients as compared to those of the healthy individuals (Quantus: median, 0.379 ng/uL versus 0.236 ng/uL; $P=0.005$; TapeStation: median,

0.224 ng/uL versus 0.143 ng/uL; $P=0.015$). However, the NanoDrop values of cancer patients did not significantly differ from those of the healthy individuals ($P=0.191$) (Fig. 2). The agreement between the two methods, Quantus and TapeStation, was then assessed. These two quantification methods were found to be significantly correlated ($r=0.802$, $P<0.001$) (Fig. 3A). The Bland-Altman plot showed a positive bias of 0.24 ng/uL for Quantus when compared with TapeStation (Fig. 3B). Interestingly, we observed that high molecular weight (HMW) DNA (>800 bp) was

present on the electropherogram of TapeStation (Fig. 4A). It is noteworthy that the difference between Quantus and TapeStation demonstrated a significantly moderate correlation with the amount of HMW DNA ($r=0.357$, $P<0.001$) (Fig. 4B).

DISCUSSION

Accurate and easily applicable strategies of cfDNA quantification are needed for cfDNA analysis. In the current study, we characterized the three different cfDNA

Fig. 1. Concentration estimates obtained using three different quantification methods, NanoDrop, Quantus, and TapeStation. The line within the box corresponds to the median. The lower limits and upper limits of the box indicate 0.25 and 0.75 quartiles, respectively.

Fig. 2. Concentration estimates obtained using three different quantification methods, NanoDrop, Quantus and TapeStation. The white bars represent the results from healthy individuals, and grey bars represent the results from cancer patients.

Fig. 3. (A) Scatter plot and (B) Bland-Altman for Quantus and TapeStation. Abbreviation: SD, standard deviation.

Fig. 4. (A) Electropherogram of cfDNA samples analyzed with high sensitivity D1000 tape of TapeStation. The distinct peak between around 170 bp represents the mono-nucleosomal fragment. The broad peak larger than 800 bp represents HMW DNA. (B) The difference in cfDNA quantification correlated with the amount of HMW DNA (ng/ μ L). Abbreviations: cfDNA, cell-free DNA; HMW, high molecular weight.

quantification methods using clinical samples. Our results showed that each method provides a different estimate of cfDNA concentration, which may be attributed to the different quantification strategies employed by each of these methods.

As expected, NanoDrop reported significantly higher concentration estimates than Quantus and TapeStation. NanoDrop estimates could not efficiently differentiate the cancer patients from healthy individuals, while those from the other two methods could do so. The high estimates of this spectrophotometric method may have occurred because of its inability to distinguish between dsDNA and ssDNA, between cfDNA fragments and protein-DNA complexes. The contaminants that absorb light at a wavelength similar to that of cfDNA may be also responsible for the overestimation by the spectrophotometer [8,9].

Quantus exhibited concentration estimates correlated to, but higher than, those of TapeStation. The difference between the two methods increased as the amount of HMW DNA increased. In this study, the DNA fragments larger than 800 bp were classified as HMW DNA [4,10]. The cfDNA presents a major peak at 166 bp corresponding to nucleosomal DNA, and the size of DNA ranges from 166 bp to approximately 500 bp according to

the mono-, di-, and tri- nucleosome structure [4,11,12]. Thus, the longer DNA fragments were considered as genomic DNA (gDNA) mostly likely originated from lysed hematopoietic cells [13,14]. Measurement of cfDNA by Quantus is based on the fluorescence intensity of fluorescent dye that directly binds to dsDNA [7]. It is important to note that the Quantus device is not able to differentiate between the size of DNA fragments [15]. On the other hand, TapeStation enables visual assessment of the distribution of fragment sizes and measures the nucleic acid concentrations for the selected size: This method has been applied in cfDNA analyses and helped to visualize the amount of distinct mono and di nucleosome DNA fragments [16-18].

Fluorometric quantification provides easy workflow and is less time consuming as compared to qPCR, therefore, a fluorometer (e.g., Qubit and Quantus) is preferred in clinical laboratories [5]. Using fluorescent probes that directly bind to dsDNA, the fluorometric quantification is an approach that compensates for the disadvantage of the spectrophotometry-based methods (e.g., NanoDrop) [19]. Several previous studies have described that the fluorometric quantification is highly correlated to qPCR quantification measurements and dPCR results [20-22]. The fragment lengths of isolated cfDNA usually are

approximately 166 bp in size, corresponding to mononucleosomal DNA. However, high molecular gDNA contamination is often observed in cfDNA isolation, which can be distinguished by electrophoresis-based methods only. The use of the electrophoresis-based method in parallel with fluorometric methods is highly recommended when quantification along with cfDNA sizing is required for NGS, as a downstream analysis [5,15,23]. The electrophoresis-based methods may also be used to assess the quality of the isolated cfDNA prior to downstream analysis, when the preanalytical conditions such as sample storage and shipping seem to be susceptible to gDNA contamination from cell lysis [24,25].

A major limitation of this study is that the reproducibility of each platform could not be demonstrated as we were not able to perform each experiment more than once. Further studies are required to investigate the analytical performance of each method. We believe that both fluorometric and electrophoresis based approaches may be used as parallel or complementary methods as mentioned above, and these technologies will influence the standardization of measuring cfDNA concentration.

ACKNOWLEDGMENTS

This work was supported by the Korean Association of External Quality Assessment Service (KEQAS) Research Fund of 2018 (2018-15).

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