Application Note

Genomics

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Accurate QC Analysis of cfDNA Using the Agilent 5200 Fragment Analyzer System

Authors

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Abstract

The increased interest in circulating cell-free DNA (cfDNA) has prompted the need for investigating the best method for evaluating cfDNA. The Agilent 5200 Fragment Analyzer system offers excellent resolution and sizing of the first three nucleosome peaks with the Agilent HS Small Fragment kit with both the Agilent FA 12-Capillary Array Short, 33 cm, and the Agilent FA 12-Capillary Array Ultrashort, 22 cm. Analysis with the Agilent HS Large Fragment kit on the 5200 Fragment Analyzer system enables reliable sizing and resolution of cfDNA containing fragmented genomic DNA with both arrays. The quickest analysis of cfDNA can be obtain with the HS Small Fragment kit with the ultrashort array.

Introduction

Circulating cell-free DNA (cfDNA) is gaining prevalence as a noninvasive, alternative approach for the detection of tumor mutations and screening tests for fetal abnormalities. cfDNA is known to circulate in healthy and pathological conditions, and is present in plasma, serum, cerebral spinal fluid, and saliva. Evidence has suggested that highly fragmented cfDNA is actively secreted when new nucleic acids are synthesized and passively released as an end product of necrosis and apoptotic cell death.

The increased use of cfDNA has prompted interest in the best evaluation method for cfDNA. In this Application Note, we compared cfDNA peak separation using two reagent kits and two different capillary arrays for the 5200 Fragment Analyzer system: HS Small Fragment kit and HS Large Fragment 50 kb kit with the FA 12-Capillary Array Short, 33 cm, and FA 12-Capillary Array Ultrashort, 22 cm. As the extraction methods play a key role in quantity and quality of cfDNA recovery, we also compared the cfDNA separation profile on the 5200 Fragment Analyzer system after extraction from two different kits.

Experimental

The experiments in this study were done using an Agilent 5200 Fragment Analyzer system and can be replicated with comparable results on Agilent 5300 and 5400 Fragment Analyzer systems.

The 5200 Fragment Analyzer system, equipped with the FA 12-Capillary Array Short, 33 cm (short array) (p/n #A2300-1250-3355) and FA 12-Capillary Array Ultrashort, 22 cm (ultrashort array) (p/n #A2300-1250-2247) was used to analyze a 1.25 or 2.5 ng/µL cfDNA sample with HS Small Fragment kit (p/n DNF-477) and HS Large Fragment 50 kb kit (p/n DNF-464). cfDNA was extracted from four healthy human serum samples (Bioreclamation IVT, #HMSRM) using the QIAmp circulating nucleic acid kit (Qiagen, #55114) or the Quick-cfDNA serum and plasma kit (Zymo, #D4076). While carrier RNA is not used in the Quick-cfDNA kit, the QIAmp kit utilized carrier RNA for the extraction of cfDNA. All serum samples extracted with the QIAmp kit were split into two 5 mL samples and extracted with and without carrier RNA to address possible carrier RNA interference with cfDNA peak separation. Some cfDNA sample was also treated with DNase to further investigate possible peak interference. A Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) was used for quantification of cfDNA.

Results and discussion

Separation of cfDNA by HS Small Fragment kit with short array

The HS Small Fragment kit distinctively separated three cfDNA peaks from healthy human serum at 157, 349, and 559 bp (Figure 1A, red trace). These peak sizes corresponded to a nucleosome-guided fragmentation pattern of apoptotic cfDNA, often referred to as mononucleosome, dinucleosome, and trinucleosome cfDNA[1]. The trinucleosome peak is not always seen and may be overshadowed by carrier RNA used in some kits. To investigate the possible interference of carrier RNA with the trinucleosome peak, DNase (Figure 1A, black trace) was added to cfDNA samples extracted with carrier RNA by the QIAmp kit. Figure 1A showed that DNase completely degraded everything in the cfDNA sample extracted with carrier RNA on the HS Small Fragment kit. The third peak detected is indeed cfDNA and not carrier RNA (Figure 1A, red trace). This was confirmed by detection of three peaks with similar base pair lengths from cfDNA extracted by the Quick-cfDNA kit, which does not utilize carrier RNA (Figure 1B).

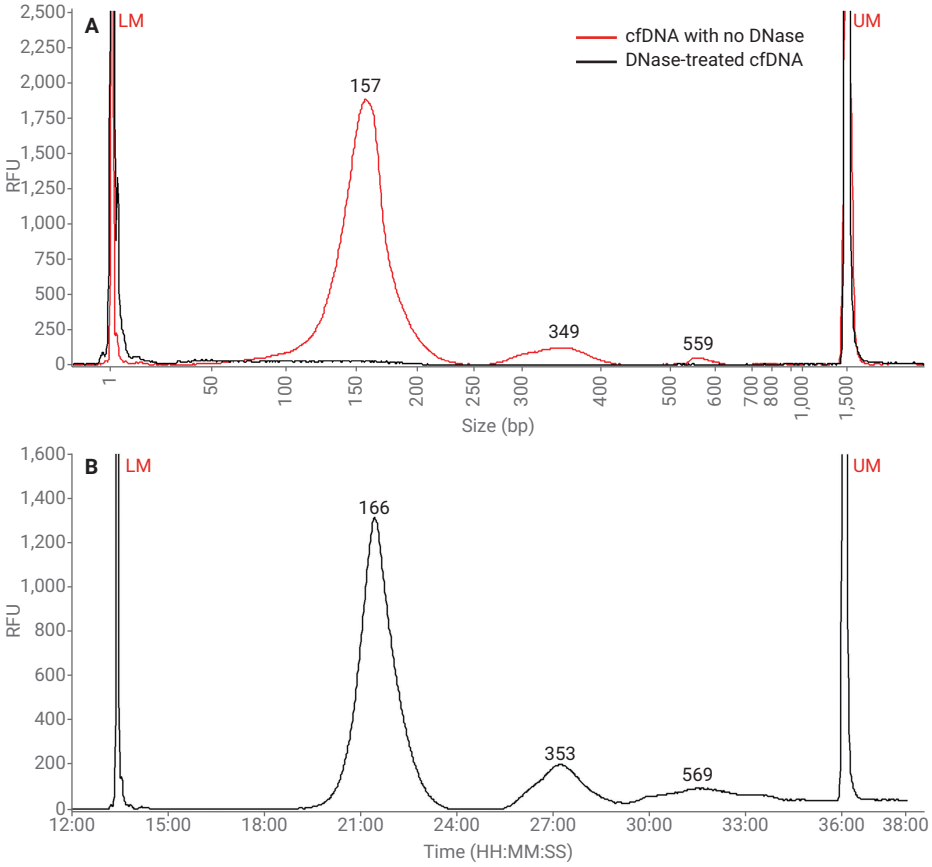

Figure 1. cfDNA separated on the Agilent 5200 Fragment Analyzer system short array with HS Small Fragment kit. (A) cfDNA extracted with the QIAmp kit using carrier RNA and treated with DNase (black trace) and without DNase (red trace). (B) cfDNA extracted without carrier RNA by the Quick-cfDNA kit. LM = lower marker, UM = upper marker

Separation of cfDNA by HS Large Fragment 50 kb kit with short array

cfDNA from sample 1 was also run using HS Large Fragment 50 kb kit and three peaks at 162, 347, and 551 bp were separated (Figure 2A, red trace). The possible interference of carrier RNA was explored by the addition of DNase to the cfDNA extracted with carrier RNA. In Figure 2A, the DNase treated cfDNA (black trace) displayed a distinct smear averaging 487 bp. Since DNase degrades only DNA, the remaining smear was likely carrier RNA. The carrier RNA smear stretched from 450 to 700 bp, overlapping with the 551 cfDNA base pair peak. These results clearly showed that carrier RNA interfered with the third cfDNA peak separation on the HS Large Fragment 50kb kit. cfDNA extracted without carrier RNA by the Quick-cfDNA kit likewise separated out three cfDNA peaks on the HS Large Fragment kit (Figure 2B). The 5200 Fragment Analyzer system detected three cfDNA peaks on both the HS Small Fragment kit and the HS Large Fragment 50 kb kit in the cfDNA samples tested. Similar separation profiles were observed for samples #1, #2, and #3, sample #1 is shown in Figures 1 and 2.

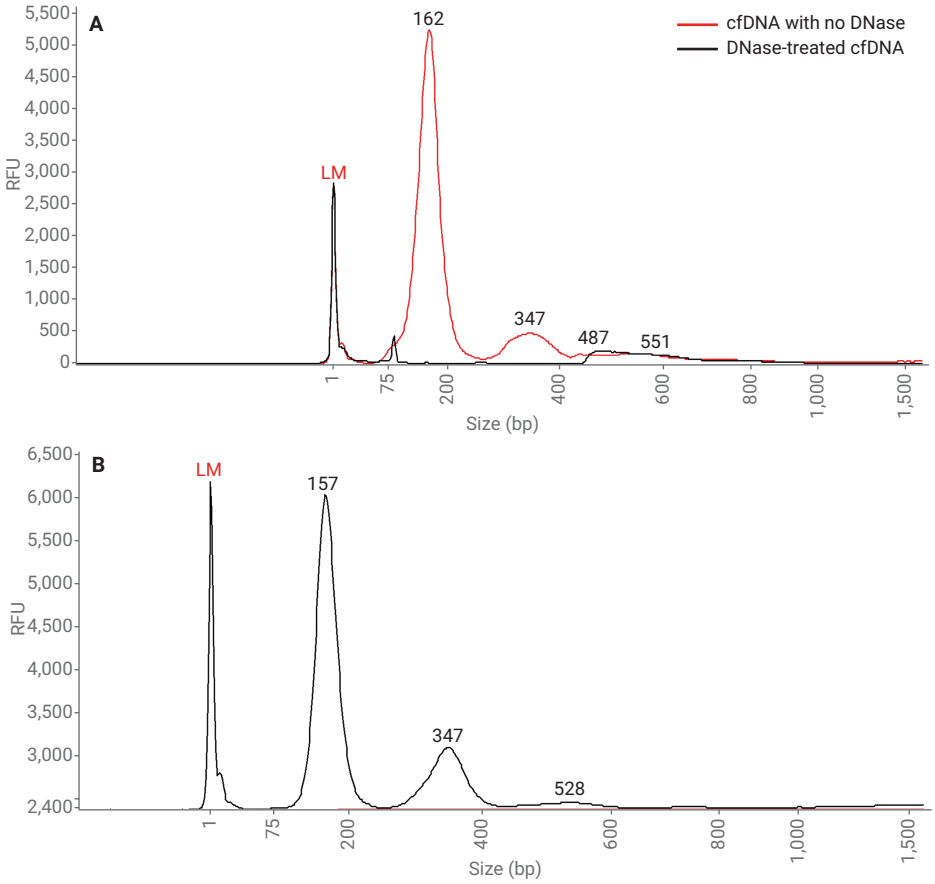

Figure 2. cfDNA separated on the Agilent 5200 Fragment Analyzer system short array with the HS Large Fragment 50 kb kit. Graphs were expanded and the upper marker cut off for comparison. (A) cfDNA extracted using the QIAmp kit with carrier RNA and treated with DNase (black trace) and without DNase (red trace). (B) cfDNA extracted without carrier RNA with the Quick-cfDNA kit. LM = lower marker

Separation of cfDNA containing gDNA

To evaluate if carrier RNA separates along with the fragmented genomic DNA, sample #4 was extracted with carrier RNA (black trace) and without carrier RNA (red trace). The results indicate that carrier RNA did not affect the profile of cfDNA when separated by the HS Small Fragment kit (Figure 3A). Sample #4 was also separated with the HS Large Fragment 50 kb kit. The HS Large Fragment 50 kb kit was capable of completely separating cfDNA with fragmented genomic DNA (Figure 3B). The genomic DNA smear occurred from 760 to 6,000 bp. However, cfDNA extracted with carrier RNA (black trace) continued to interfere with the separation of the 582 bp cfDNA peak on the HS Large Fragment 50 kb kit.

The separation profile of cfDNA from sample #4 using the HS Small Fragment kit is shown in Figure 3A. In addition to the three standard nucleosome peaks, sample #4 contained fragmented genomic DNA. The smear started at 800 bp and ran through the 1,500 bp upper marker (Figure 3A). An extended run time was utilized to complete the DNA separation with the HS Small Fragment kit.

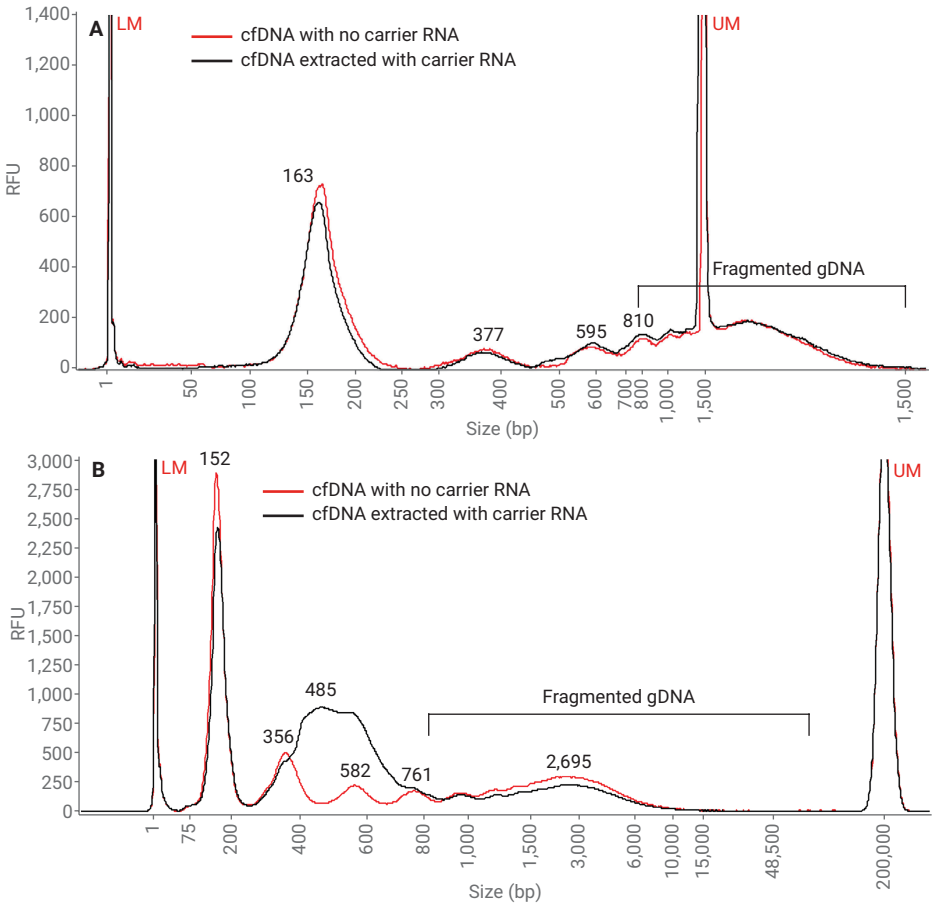

Figure 3. cfDNA sample #4 containing fragmented genomic DNA was separated on the Agilent 5200 Fragment Analyzer system short array. (A) cfDNA extracted with carrier RNA (black trace) and without carrier RNA (red trace) with the HS Small Fragment kit. (B) cfDNA extracted with carrier RNA (black trace) and without carrier RNA (red trace) with the HS Large Fragment 50 kb kit. LM = lower marker, UM = upper marker

Comparison of different cfDNA extraction kits

The concentration of cfDNA found in serum samples is extremely low. Therefore, utilizing the most effective extraction kit is a necessity. We compared the total cfDNA extraction yield from 5 mL of healthy human serum between the QIAmp kit and the Quick-cfDNA kit (Table 1).

Table 1. Comparison of the total cfDNA yield from the QIAmp kit and the Quick-cfDNA kit.

Healthy human serum[1]			
	Volume of sample	Average extracted quantity (ng)	Suspension volume (µL)
QiAmp kit with carrier RNA	5 mL	70	20
Quick-cfDNA kit	5 mL	136	50

[1] n = 4 from same sample

Comparison between short array and ultrashort array

The ultrashort array was utilized to reduce analysis time of cfDNA extracted by the Quick-cfDNA kit. The separation time was reduced by 10 minutes for the HS Small Fragment kit (Figure 4A) and by 15 minutes for the HS Large Fragment 50 kb kit (Figure 4B). Both kits produced excellent separations of the cfDNA peaks on the 5200 Fragment Analyzer system ultrashort array.

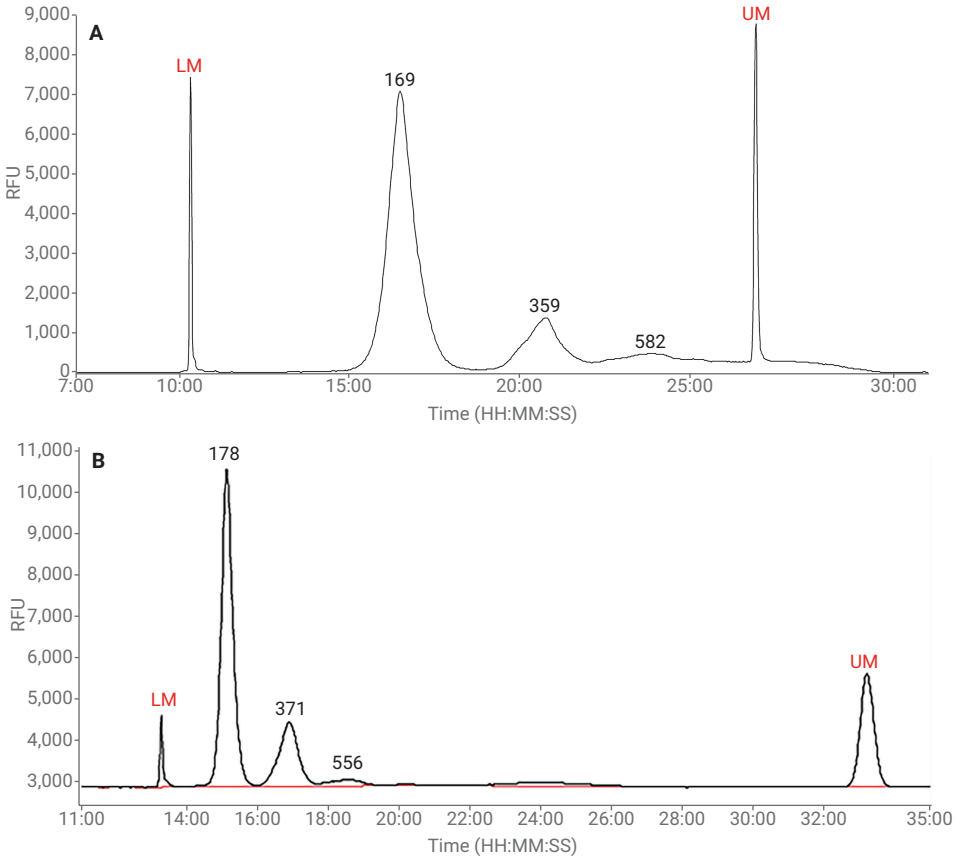

Figure 4. cfDNA extracted with the Quick-cfDNA kit and separated on the Agilent 5200 Fragment Analyzer system ultrashort array. (A) HS Small Fragment kit. (B) HS Large Fragment 50 kb Kit. LM = lower marker, UM = upper marker

Conclusions

Table 2 summarizes the differences between the HS Small Fragment kit and the HS Large Fragment 50 kb kit for cfDNA analysis on the Fragment Analyzer system. The HS Small Fragment kit provided efficient and excellent separations of cfDNA extracted with or without carrier RNA, however it could not completely separate the fragmented genomic DNA. The HS Large Fragment 50 kb kit completely separated cfDNA that contained fragmented genomic DNA but displayed interference from carrier RNA. When possible, cfDNA extracted without carrier RNA is recommended to avoid carrier RNA interference.

Table 2. Comparison of cfDNA analysis methods on the 5200 Fragment Analyzer system.

	Fragment Analyzer short array run time	Fragment Analyzer ultrashort array run time	Comments
HS Small Fragment kit 50 to 1,500 bp	40 minutes	30 minutes	• Good separation of cfDNA • No effect from extraction carrier RNA • Fragmented genomic DNA merged with upper marker
HS Large Fragment kit 75 to 50,000 bp	50 minutes	35 minutes	• Good separation of cfDNA • Interference from extraction carrier RNA • Good separation of fragmented genomic DNA

Reference

1. Suzuki, N.; *et al.*, Characterization of circulating DNA in healthy human plasma, *Clinica Chimica Acta* **2008**, *387*, 55–58.

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