

# cfDNA Separated on the Agilent Femto Pulse System

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## Abstract

Circulating cell-free DNA (cfDNA) is gaining prevalence as a non-invasive liquid biopsy approach for screening of health-related issues. The Agilent Femto Pulse system and the Agilent Ultra Sensitivity NGS kit offer unparalleled resolution and quality assessment of low concentrated cfDNA samples with the capability of separating up to seven different cfDNA fragments.

## Introduction

Circulating cell-free DNA (cfDNA) are small DNA fragments found circulating in plasma, serum, saliva, and other bodily fluids. Currently, sizing and concentration of cfDNA, along with detection of mutations, are being researched in many health-related issues. Separation of cfDNA on the Agilent Femto Pulse system with the Agilent Ultra Sensitivity NGS kit displays a mono-nucleosome fragment around 170 bp, a di-nucleosome fragment around 350 bp and a tri-nucleosome fragment between 550 to 580 bp. Larger cfDNA fragments are also often present in regular intervals of 180 to 200 bp. cfDNA samples can differ in the number of fragments present, but the mono- and di-nucleosome fragments are always present. The Femto Pulse offers remarkably consistent sizing, quantification, and resolution of all the cfDNA fragments at low picogram concentrations.

## Experimental

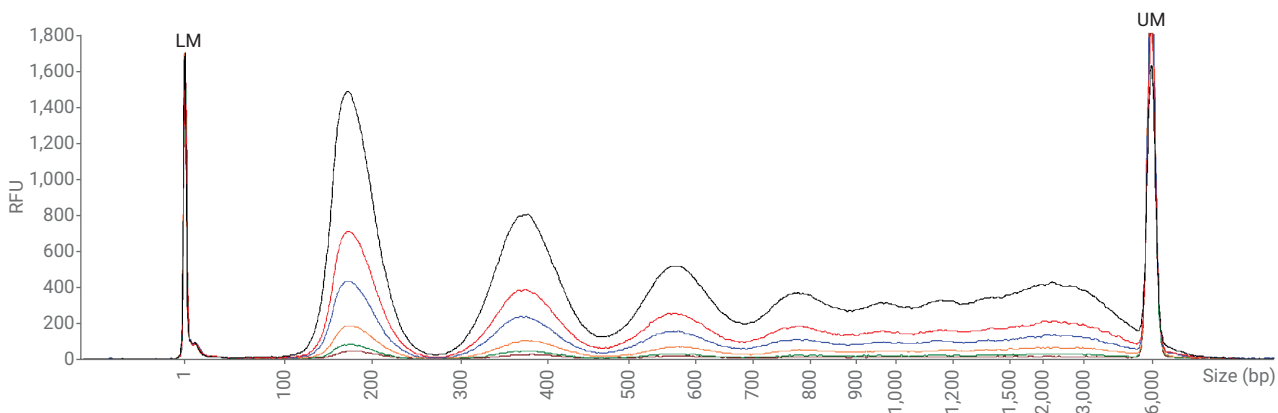
Several different cfDNA samples were extracted from healthy human serum samples (#HMSRM) from Bioreclamation IVT using the Quick-cfDNA serum and plasma kit (Zymo, #D4076). The Agilent Femto Pulse system equipped with the Agilent Femto Pulse 12-Capillary Array (ultrashort array) (p/n A1600-1250-2240) was used to analyze various concentrations of cfDNA with the Agilent Ultra Sensitivity NGS kit (FP-1101). An extended prerun (-3 kV; 2,700 s) was utilized for optimal analysis. cfDNA samples were diluted in either 0.25x TE buffer or Qubit dsDNA HS buffer (Thermo Fisher Scientific, #Q32851).

## Results and discussion

Separation of cfDNA on the Femto Pulse (Figure 1) allows for easy visualization of up to seven cfDNA fragments. In addition, the Agilent 5200 Fragment Analyzer system is capable of separating multiple cfDNA fragments. The knowledge that cfDNA has fragments longer than the mono-, di-, and tri-nucleosome, opens up opportunities and creates possibilities for future research. The ability to reliably visualize the entire cfDNA sample for quality control ensures the best possible outcome for research involving amplification and sequencing results. This new information about cfDNA discovered with progressive Agilent technology has widespread potential for furthering current research in genetic changes, structural variance, and providing insight into disease progression.

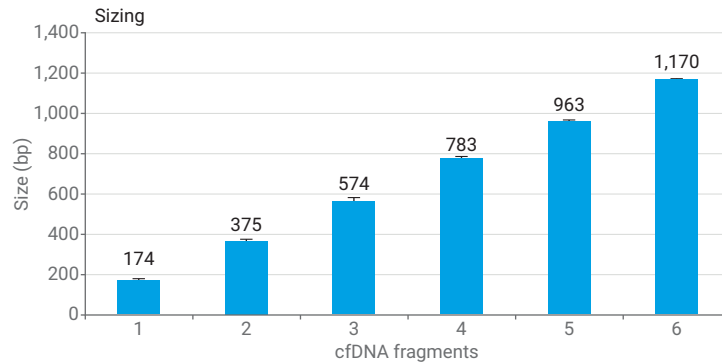
### Concentration range and sizing

cfDNA was analyzed on the Femto Pulse with the Agilent Ultra Sensitivity NGS kit, which has a DNA smear detection range of 5 to 250 pg/ $\mu$ L. A dilution series (250, 125, 62.5, 31.3, 15.6, 7.8 pg/ $\mu$ L) was performed to determine a concentration range ideal for the separation of cfDNA on the Femto Pulse (Figure 1).

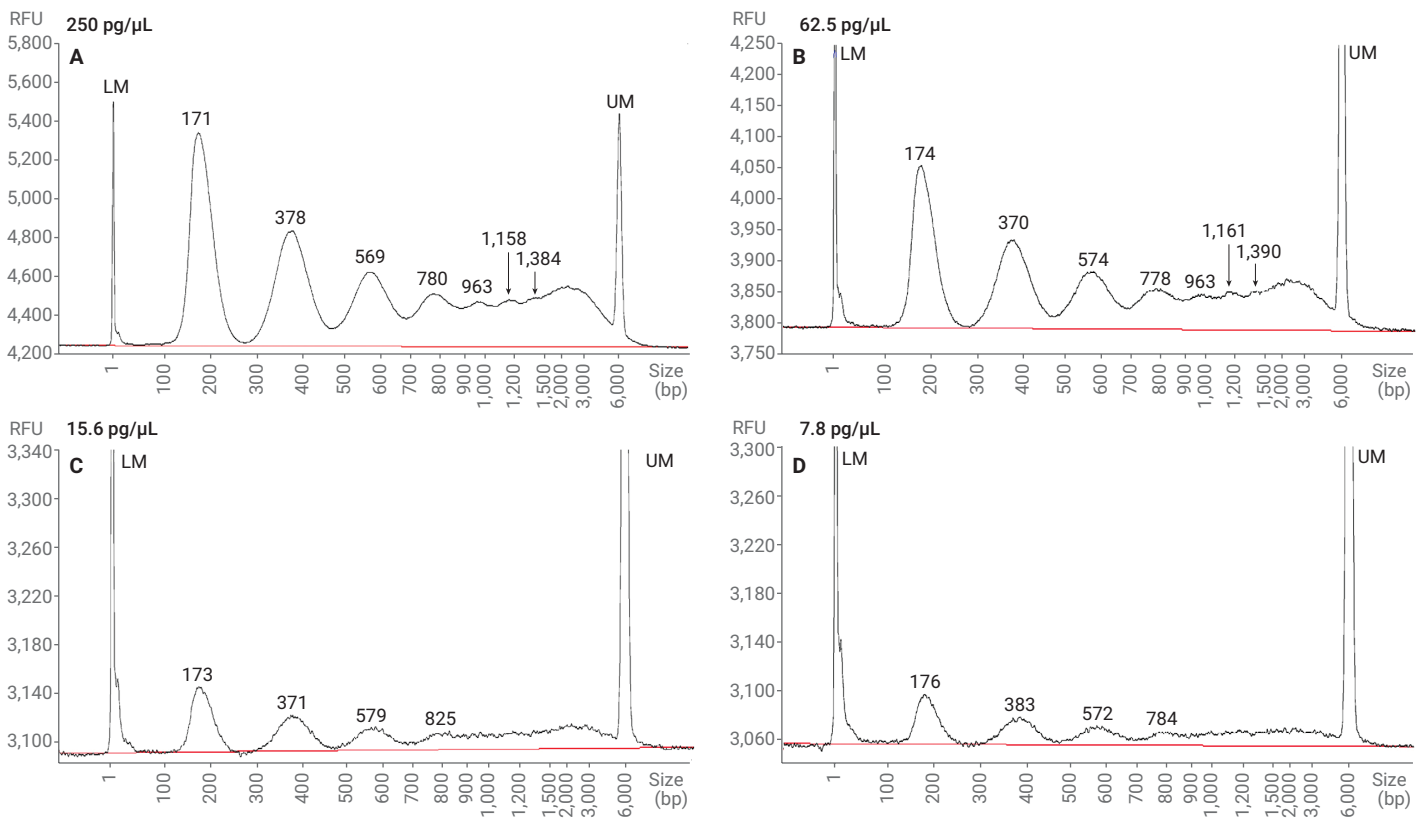


**Figure 1.** Overlay of cfDNA dilution series on the Agilent Femto Pulse system with the Agilent Ultra Sensitivity NGS kit with extended prerun (250, 125, 62.5, 31.3, 15.6, 7.8 pg/ $\mu$ L). LM = lower marker; UM = upper marker.

The first 3 cfDNA fragments were completely separated at all concentrations, with the fourth fragment easily distinguishable down to 15.6 pg/μL (Figure 2). The fifth and sixth fragment peaks were less apparent below 31.3 pg/μL. A seventh fragment peak was observed only in the highly concentrated samples, 62.5 pg/μL and higher, with an average size of 1,387 bp. Sizing remained consistent for the six cfDNA fragments across the concentration range with a % CV less than 1.3 % (Figure 3)



**Figure 3.** Average size of the cfDNA fragments across the dilution range of 7.8 to 250 pg/μL. n=21.



**Figure 2.** cfDNA separated on the Agilent Femto Pulse system with the Agilent Ultra Sensitivity NGS kit with extended prerun. (A) 250 pg/μL; (B) 62.5 pg/μL; (C) 15.6 pg/μL; (D) 7.8 pg/μL. LM = lower marker; UM = upper marker.

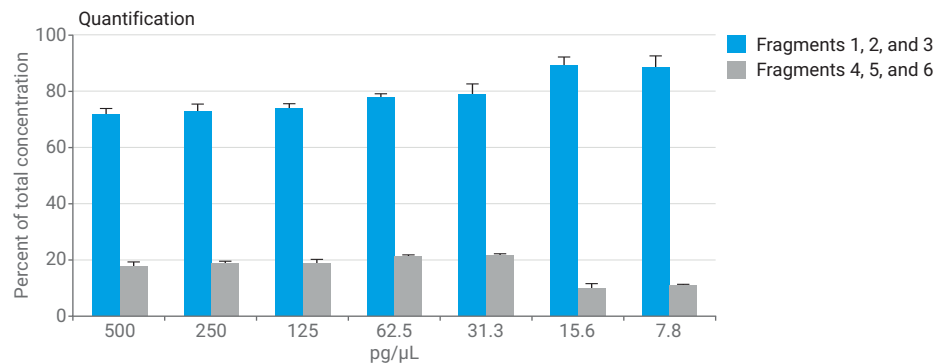
## Quantification

Commonly, only the combined concentration of the mono-, di-, and tri-nucleosome cfDNA fragments is utilized when comparing and determining healthy from diseased states. Healthy individuals have been shown to have a lower concentration of the first three cfDNA fragments in plasma and serum compared to diseased states. Analysis of the cfDNA dilution series (Figure 4) showed that the percent of total concentration of the first three fragments combined and the fourth, fifth, and sixth fragments combined remained consistent ( $76\% \pm 1.8$  and  $20\% \pm 0.8$ , respectively) over the sample concentration range of 31.3 to 250  $\text{pg}/\mu\text{L}$ . The percent of total concentration increased for the first three fragments to 89% at the lower concentrations (7.8 to 15.6  $\text{pg}/\mu\text{L}$ ) due to undetectable amounts of the fifth and sixth cfDNA fragments.

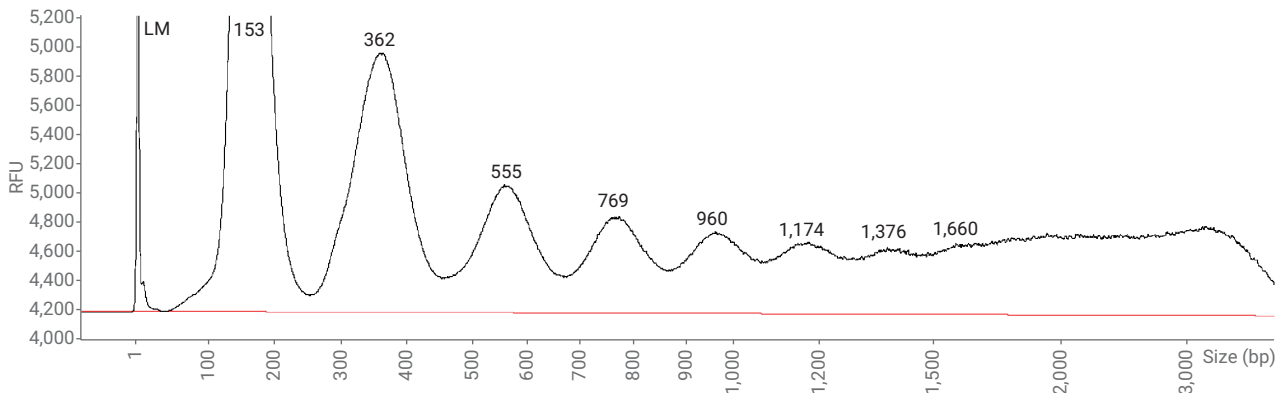
## Separation parameters

To evaluate if all possible cfDNA fragments have been separated and visualized, an experimental kit with no upper marker and an experimental method was explored on the Femto Pulse. Removal of the upper marker eliminates any separation interference with the larger cfDNA

fragments. Excellent visualization and separation of seven, and possibly eight, cfDNA fragments was achieved for demonstration purposes only, with the following method: sample injection (-5 kV, 30 s); separation voltage (-1 kV, 240 min) (Figure 5). This experimental method displayed better resolution between the larger cfDNA fragments.



**Figure 4.** Average of the percent of total concentration of the combined mono-, di-, tri-nucleosome and the combined fourth, fifth, and sixth cfDNA fragments across the dilution range of 7.8 to 250  $\text{pg}/\mu\text{L}$ .  $n=21$ .



**Figure 5.** For demonstration purposes only, cfDNA was separated on the Agilent Femto Pulse system with an experimental kit and method for enhanced separation of the larger cfDNA fragments. LM = lower marker.

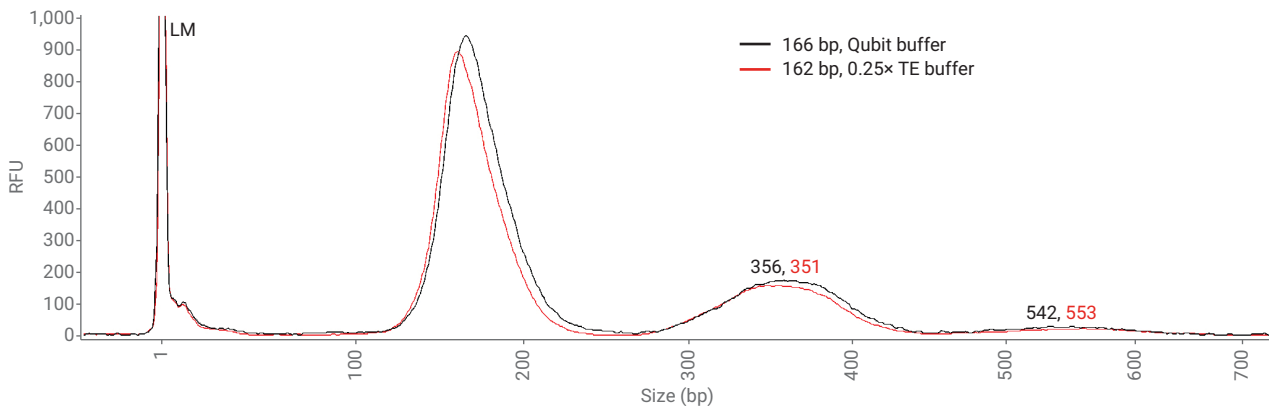
## Conservation of sample

cfDNA is usually extracted in small quantities. As a result, the amount of sample available for quality control analysis is limited. Even though the Agilent Femto Pulse can be utilized directly for qualitative and quantitative analysis of cfDNA, often the concentration of the sample is determined prior to analysis with a fluorometric instrument. The high detection sensitivity of the Agilent Femto Pulse allows samples diluted in the dsDNA HS Qubit buffer to be directly

analyzed on the Femto Pulse, thereby conserving sample.

To determine if cfDNA prepared in Qubit dsDNA HS buffer would affect the separation on the Femto Pulse, cfDNA was diluted in Qubit dsDNA HS buffer or 0.25x TE buffer to reach a range of calculated concentrations based on Qubit measurements (4.38 to 35 pg/ $\mu$ L). The samples were then separated on the Agilent Femto Pulse following the Agilent Ultra Sensitivity NGS kit protocol (Figure 6). Sizing of the mono-, di-, and tri-nucleosome

fragments remained consistent over the range of concentrations and in both the 0.25x TE and Qubit dsDNA HS buffer (Table 1). Quantification was similar to the expected diluted concentration and comparable between the two buffers over the range of concentrations. Analyzing samples directly from the Qubit dsDNA HS buffer may result in lower concentrations that only allow for the visualization of the mono-, di-, and tri-nucleosome fragments.



**Figure 6.** cfDNA (35 pg/ $\mu$ L) diluted with Qubit dsDNA HS buffer (black) or 0.25x TE buffer (red) and separated on the Agilent Femto Pulse system with the Ultra Sensitivity NGS kit. Average size, n=3. LM = lower marker.

**Table 1.** Sizing and quantification of cfDNA diluted in 0.25x TE buffer or Qubit dsDNA HS buffer and separated on the Agilent Femto Pulse system with the Agilent Ultra Sensitivity NGS kit. n=3.

	Expected diluted concentration (pg/ $\mu$ L)	TIC (pg/ $\mu$ L)	Percentage of total concentration (%)			Peak size (bp)		
			Mononucleosome	Dinucleosome	Trinucleosome	Mononucleosome	Dinucleosome	Trinucleosome
Qubit buffer	35	38.49	74.9	22.5	2.6	166	356	542
	17.5	18.98	72.3	23.6	4.1	167	357	540
	8.75	7.64	74.9	23.7	2.1	181	384	565
	4.38	4.65	77.7	21.4		176	377	
0.25x TE	35	37.30	74.5	22.9	2.7	162	351	553
	17.5	22.18	74.3	23.2	2.5	162	356	562
	8.75	10.30	76.8	22.6	1.8	161	362	566
	4.38	4.96	76.3	23.7		161	353	

## Conclusions

The Agilent Femto Pulse system can visualize up to seven cfDNA fragments allowing for the potential to gather more information and insight for future research. The recommended concentration range for cfDNA analysis on the Agilent Femto Pulse with the Agilent Ultra Sensitivity NGS kit with an extended prerun is 31.3 to 250 pg/ $\mu$ L. This range allows for visualization of seven cfDNA fragment peaks with accurate sizing and consistent quantification for the first six fragments. At concentrations lower than 31.3 pg/ $\mu$ L, sizing and quantification analysis of the first three cfDNA fragments remains accurate and precise. Conservation of cfDNA samples can be achieved by analyzing cfDNA diluted in Qubit dsDNA HS buffer on the Femto Pulse with the Agilent Ultra Sensitivity NGS kit. Analysis of cfDNA in the Qubit dsDNA HS buffer provides consistent and similar sizing when compared to dilution in the recommended 0.25 $\times$  TE buffer.

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