Technical Overview

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Detection of Contaminating High Molecular Weight DNA with the Cell-Free DNA ScreenTape Assay

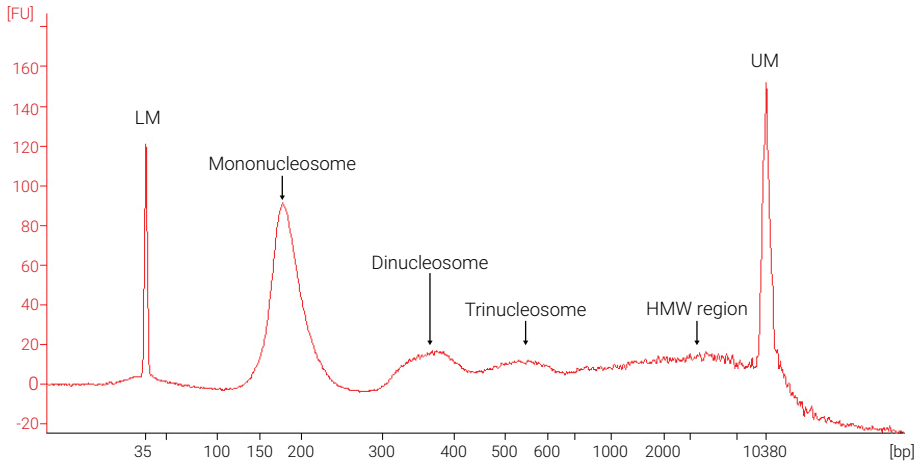
Introduction

Cell-free DNA (cfDNA) has become an essential tool in clinical research, enabling identification of potential biomarkers and mutations via noninvasive collection methods. For example, biofluids such as plasma, serum, urine, and cerebrospinal fluid often contain cfDNA, released from apoptotic cells. However, cfDNA represents a challenge for next-generation sequencing (NGS) workflows due to the low yield, complex fragmentation pattern, and possibility of contaminating high molecular weight (HMW) DNA. Assessment of cfDNA prior to downstream genetic analysis is thus crucial to ensuring the sample is of sufficient quality for successful workflows.

To appropriately assess the quality of cfDNA, it is important to visualize the fragmentation pattern and any degradation or contamination within the sample. Electrophoretic methods are ideal for this assessment. Typically, cfDNA consists of many fragments, including the DNA that is wrapped around nucleosomes, different lengths of DNA linking the nucleosomes together, and degradation products. Electrophoretic separation of cfDNA is thus often visualized as multiple peaks, displaying the mono-, di-, and tri-nucleosomal fragments. Qualitative and quantitative assessment of the fragments can be performed using Agilent automated electrophoresis instruments, including the Bioanalyzer and TapeStation systems, as shown in Figure 1.

Along with visualization of the fragments, cfDNA samples should also be assessed for the presence of HMW DNA contamination, which can lead to misrepresentation of the total sample concentration and negatively affect NGS library yield and sequencing results. For highly pure cfDNA that is lacking HMW DNA, analysis with the Agilent 2100 Bioanalyzer system and the Agilent High Sensitivity DNA kit provides appropriate evaluation of the fragmentation pattern. However, when HMW DNA is present, the upper marker used for sizing and quantitation with this assay may overlap with the HMW DNA contamination and can impact assessment of the total sample. As the amount of HMW DNA can vary depending on extraction method and sample origin, it is important that the method used for cfDNA analysis is not impacted by an upper marker. To accomplish this, Agilent developed the Cell-free DNA ScreenTape assay for the TapeStation systems. The assay calculates a %cfDNA quality score for objective assessment of the amount of cfDNA in the sample compared to any HMW DNA contamination[1,2]. The %cfDNA score provides valuable information for screening the quality of input cfDNA samples prior to NGS library preparation and sequencing[2]. This technical overview compares the analysis of cfDNA contaminated with HMW DNA between the Bioanalyzer and TapeStation systems.

A. Agilent 2100 Bioanalyzer system



B. Agilent 4200 TapeStation system

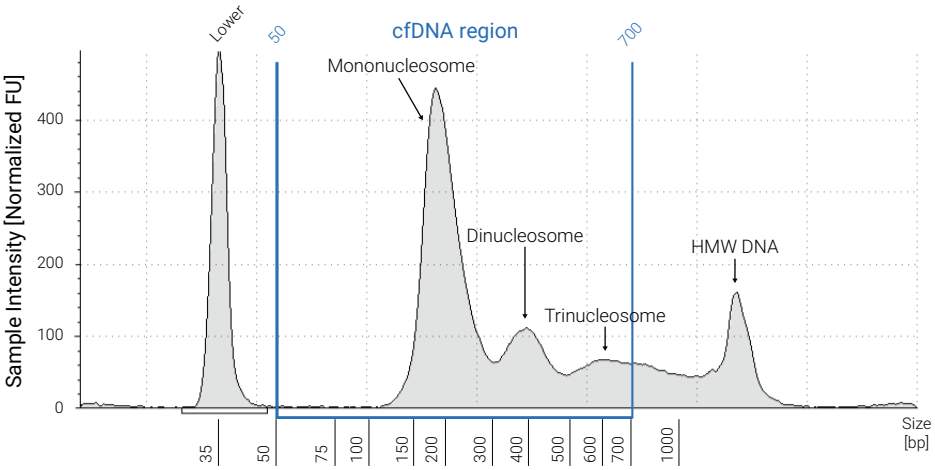


Figure 1. Representative electropherogram images of the same cfDNA sample analyzed using A) the Agilent Bioanalyzer system with the Agilent High Sensitivity DNA kit and B) an Agilent 4200 TapeStation system with the Agilent Cell-free DNA ScreenTape assay. Both systems allow for visualization of the cfDNA fragmentation pattern, while the absence of the upper marker in the TapeStation assay provides for better visualization of the HMW region, which overlaps with the upper marker in the Bioanalyzer assay. The predefined cfDNA region of 50 to 700 bp on the cfDNA assay is noted by the blue lines and is used to calculate the %cfDNA quality score. LM: lower marker, UM: upper marker, HMW: high molecular weight.

Experimental

Several independent plasma samples were processed with different extraction kits. cfDNA was extracted from human blood plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen p/n 55114), MagMax Cell-Free DNA Isolation kit (Thermo Fisher Scientific p/n A29319), the NucleoSnap cfDNA Plasma kit (Macherey-Nagel p/n 740300.10), and the Mag-Bind cfDNA kit (Omega Bio-tek p/n M3298-01). The extracted cfDNA samples were analyzed with the Agilent Cell-free DNA ScreenTape (p/n 5067-5630) and Agilent Cell-free DNA reagents (p/n 5067-5631) in combination with the Agilent 4200 TapeStation system. For comparison, the samples were also analyzed with the Agilent High Sensitivity DNA kit (p/n 5067-4626) using the Agilent 2100 Bioanalyzer system.

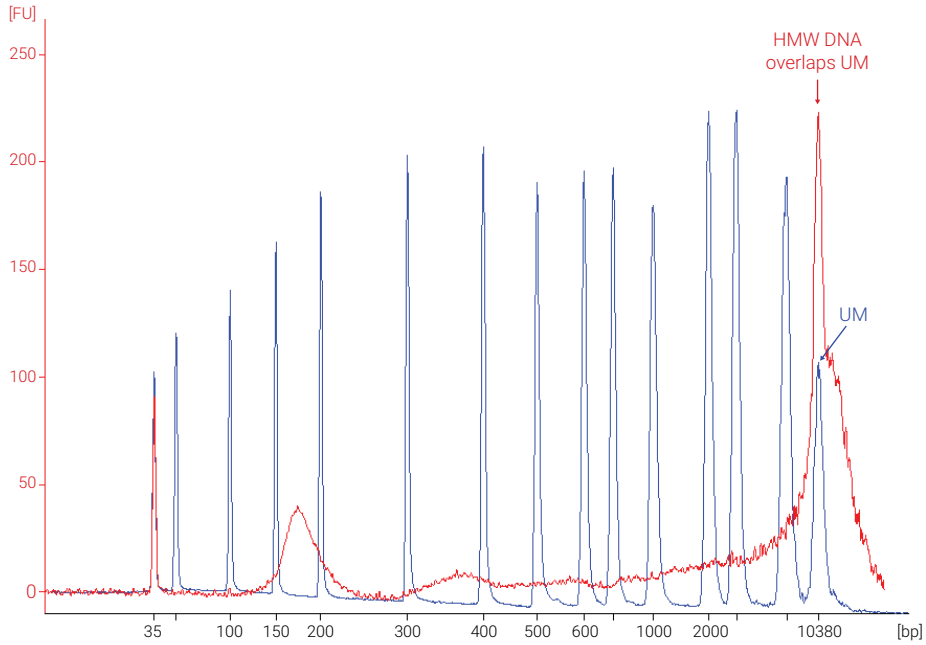
Results and discussion

Visualization of cfDNA

HMW DNA contamination is often seen in cfDNA extractions but can interfere with downstream processes that require highly pure cfDNA. As shown in Figure 1, both the Bioanalyzer and TapeStation systems are suitable for visualization of cfDNA when there is little to no HMW DNA contamination. However, HMW DNA can interfere with analysis of cfDNA on the Bioanalyzer, oftentimes extending over a large sizing range and overlapping with the upper marker that is crucial for accurate sizing and quantification. Quality assessment of cfDNA thus requires visualization of the nucleosomal peaks, as well as any contaminating HMW DNA that may exist in the sample.

Figure 2 shows an overlay of the same sample with the kit-specific ladder and markers for the Bioanalyzer (A) and TapeStation (B) to demonstrate the differences between the two systems. With the Bioanalyzer and the HS DNA kit, the last peak seen in the ladder is the upper marker at 10,380 bp. The HMW contamination in the sample overlaps the upper marker, causing a broad peak that is incorrectly assigned by the software, thus impacting size and quantitative analysis of the cfDNA sample (Figure 2A). Alternately, the Cell-free DNA ScreenTape assay for the TapeStation does not use an upper marker, and an overlay of the same sample with the ladder shows that the HMW DNA is visualized after the cfDNA fragments and the last ladder peak (Figure 2B). The absence of an upper marker with the TapeStation assay allows for more accurate analysis of the cfDNA apart from the HMW DNA contamination.

A. Agilent 2100 Bioanalyzer system



B. Agilent 4200 TapeStation system

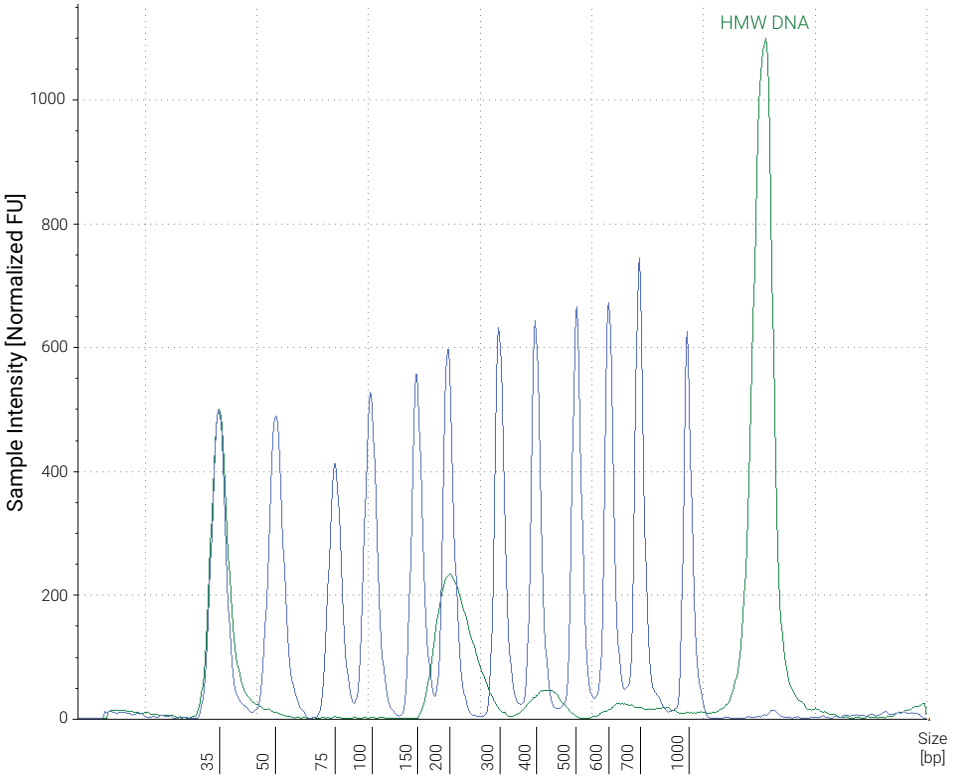


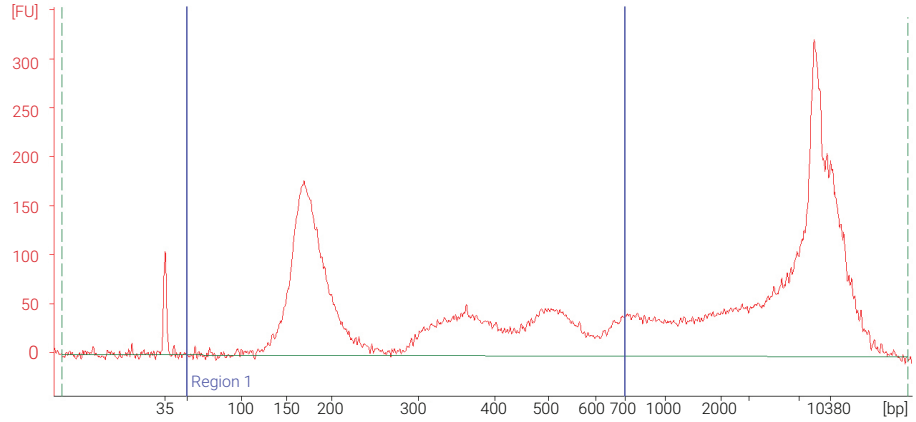
Figure 2. A representative cfDNA sample overlaid with the ladders and markers specific for A) the Agilent High Sensitivity DNA kit for the Bioanalyzer system, and B) the Agilent Cell-free DNA ScreenTape assay for the TapeStation systems. This sample contains a large amount of HMW DNA contamination which overlaps with the upper marker in the Bioanalyzer electropherogram but is separated apart from the TapeStation cfDNA ladder. UM: upper marker, HMW: high molecular weight.

Quality assessment of cfDNA with the %cfDNA quality metric

The Cell-free DNA ScreenTape assay for the TapeStation has been optimized for accurate qualitative and quantitative analysis of cfDNA. The assay utilizes a preset region analysis (50 to 700 bp) to define the cfDNA area, making it possible to accurately assess the cfDNA region apart from the HMW region. A %cfDNA quality metric score is assigned to each sample to easily identify the portion of the sample that is composed of the subunits of cfDNA in relation to any HMW DNA contamination that may be present. For example, as shown in Figure 1B, the cfDNA region is highlighted between the blue region analysis lines. The peak to the right of the cfDNA region is HMW DNA. When analyzed on the TapeStation, this sample had a %cfDNA score of 76%, indicating that a large portion of the sample is composed of cfDNA fragments.

In another example, Figure 3 shows a sample with a large HMW DNA peak. As shown in Figure 3A, it is possible to set a region analysis for the samples analyzed on the Bioanalyzer and visualize the cfDNA peaks, but the presence of HMW DNA distorts the upper marker, negatively influencing cfDNA quantification. In contrast, the preset cfDNA region applied by the TapeStation analysis software allows for quick and accurate assessment of the cfDNA apart from the large HMW DNA contamination and displays a %cfDNA score of 41% for this sample (Figure 3B).

A. Agilent 2100 Bioanalyzer system



B. Agilent 4200 TapeStation system

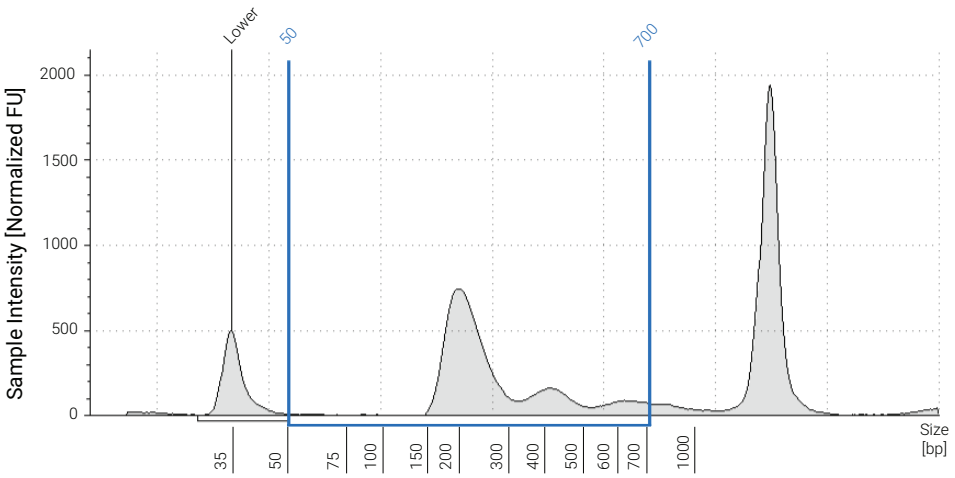


Figure 3. A cfDNA sample with a large amount of HMW DNA contamination was analyzed on the A) Agilent 2100 Bioanalyzer system and B) Agilent 4200 TapeStation system. The large amount of HMW DNA corresponds to a %cfDNA score of 41% upon analysis with the TapeStation using the Cell-free DNA ScreenTape assay.

The TapeStation system provides both an electronic gel image and an electropherogram of each sample. For the purposes of this study, ten samples with varying amounts of HMW contamination were examined in triplicate. The average %cfDNA score for each sample is shown in Figure 4A, and the average concentration for the cfDNA region compared to the total DNA is shown in Figure 4B. The %CV of the %cfDNA score for each sample was less than five, indicating excellent precision between replicates. Figure 4C shows an electropherogram overlay of different cfDNA samples with varying %cfDNA scores. In these examples, the amount of cfDNA stays consistent, while the quantity of HMW contamination changes. The total DNA quantification of the cfDNA samples is overestimated due to the presence of HMW DNA in the sample (Figure 4B). For fast and automated qualification of multiple samples, the %cfDNA value is depicted below the gel image (Figure 4D) and is color-coded to indicate high (green), medium (yellow), and low (red) quality samples. The %cfDNA region and the %cfDNA thresholds indicating quality are preset with the assay, but can be customized to fit specific user needs.

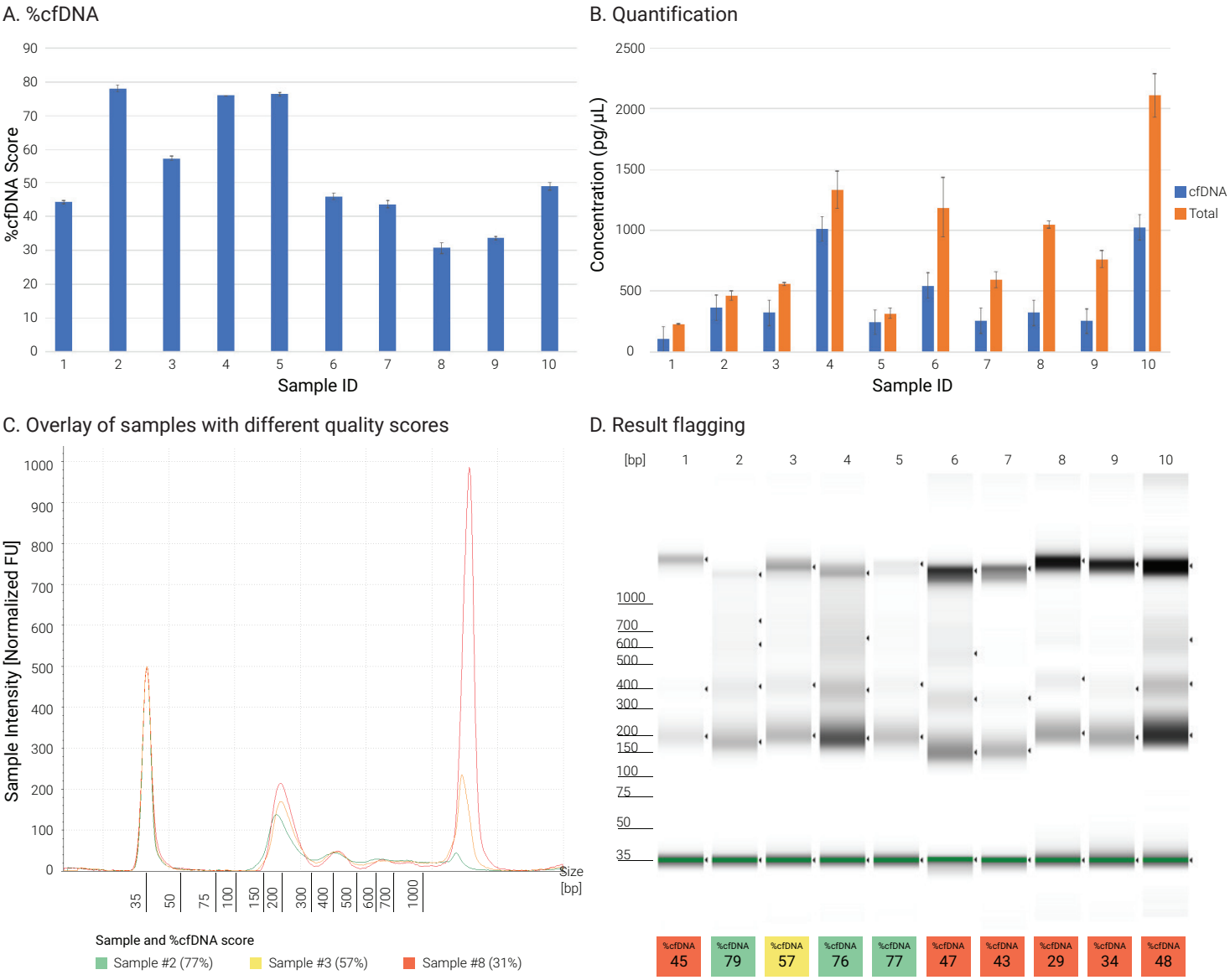


Figure 4. Ten cfDNA samples were analyzed on the Agilent TapeStation system with the Agilent Cell-free DNA ScreenTape assay. A) The average %cfDNA score and B) concentration of each sample (n = 3; error bars are standard deviation). C) Electropherogram overlay comparison of samples with different quality scores. D) Example of digital gel image results, with the %cfDNA score and user-defined color coding to indicate high (green), medium (yellow), or low (red) quality thresholds below the sample for quick assessment of multiple samples.

Conclusion

Quality control of cfDNA is essential to the success of downstream workflows. Electrophoretic separation of cfDNA samples allows for assessment of the total sample, including both the portion of the sample that is composed of cfDNA and any HMW DNA contamination. When quantified with techniques such as UV-Vis and fluorometric methods, the presence of HMW DNA within a cfDNA sample can artificially increase the concentration of the cfDNA, as there is no way to distinguish the cfDNA fragments from the HMW DNA. This can then result in too little sample being used for NGS library preparation, and thus negatively impact sequencing results.

Both the Agilent 2100 Bioanalyzer and Agilent TapeStation systems provide visualization of cfDNA. While the Bioanalyzer can be used for analysis of highly pure cfDNA, it does utilize an upper marker for sizing that can overlap with HMW DNA present in the sample, impacting sizing and quantification of the sample. For objective and reliable assessment of a sample, in combination with a dedicated quality metric for cfDNA, analysis with the Agilent Cell-free DNA ScreenTape assay using the TapeStation is the ideal solution. A preset region from 50 to 700 bp separates the cfDNA from any high molecular weight DNA present, allowing for quantification of the total sample or the cfDNA region alone, thereby enabling appropriate assessment of the cfDNA content within the sample. The assay also provides each sample with a %cfDNA quality score, which determines the percentage of the sample in the cfDNA region apart from any HMW contamination, allowing for rapid assessment of sample quality.

References

1. Performance Characteristics of the Cell-Free DNA ScreenTape Assay. *Agilent Technologies technical overview*, publication number 5994-1390EN, **2019**.
2. Quality Control of Cell-free DNA Samples Analyzed with Next-Generation Sequencing. *Agilent Technologies application note*, publication number 5994-2284EN, **2020**.

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