Technical Overview

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Performance Characteristics of the Cell-free DNA ScreenTape Assay

Introduction

Cell-free DNA (cfDNA) has become an important biomarker for health-related issues. The development of low-input library protocols for next-generation sequencing (NGS) workflows now enables the sequencing of cfDNA and improves the likelihood of identifying new mutations and biomarkers. Yet, cfDNA still represents the most challenging starting material used for NGS workflows due to its low yield and multiple fragment sizes. Sample extraction and pre-analytical treatments require a high level of care and standardization to obtain cfDNA samples with sufficient yield and adequate quality for sequencing. Traditionally, fluorescent methods were used to determine the concentration of cfDNA samples. These methods are limited and only provide information about total DNA yield, without differentiation of the cfDNA subcomponents or the possible presence of high molecular weight (HMW) DNA. The presence of HMW DNA in cfDNA samples can negatively affect library yield and sequencing quality. The Agilent Cell-free DNA ScreenTape assay was developed for cfDNA analysis, providing reliable total DNA concentration as well as quantitative assessment of cfDNA subcomponents apart from HMW DNA. Also, the assay provides a %cfDNA metric to qualify cfDNA samples by reporting the percent of cfDNA subcomponents compared to the total sample present. The Cell-free DNA ScreenTape assay is used with the Agilent 4150 or 4200 TapeStation systems, offering all the benefits of fully automated electrophoresis for fast and reliable analysis, with ready-to-use consumables and minimal handling.

This Technical Overview focuses on the performance of the Cell-free DNA ScreenTape assay by evaluating sensitivity, accuracy, and precision of quantification and sizing, while exploring the performance of the new quality metric %cfDNA. Performance of the assay was compared on the 4150 and the 4200 TapeStation systems to demonstrate seamless transition between the two instruments.

Analytical Specifications

The analytical specifications of the Agilent Cell-free DNA ScreenTape assay for both the 4150 and the 4200 TapeStation systems are summarized in Table 1.

Table1. Analytical specifications of the Agilent Cell-free DNA ScreenTape assay.

Analytical Specifications	Cell-Free DNA ScreenTape Assay
Sizing range	50 to 800 bp
Sensitivity[1]	20 pg/µL
Sizing precision[2]	10% CV
Sizing accuracy[2, 3]	± 15%
Quantitative precision[2]	15% CV
Quantitative accuracy[2]	± 20%
Quantitative range	100 to 4,000 pg/µL
%cfDNA functional range	100 to 5,000 pg/µL

[1] Signal-to-noise >3 (single peak)
[2] Determined using the Agilent Cell-free DNA ladder as sample
[3] Sizing accuracy for analysis with electronic ladder ± 20%

Experimental

Materials

The Agilent 4150 TapeStation system (p/n G2992AA) and the Agilent 4200 TapeStation system (p/n G2991AA) with the Agilent Cell-free DNA ScreenTape (p/n 5067–5630) and Agilent Cell-free DNA reagents (p/n 5067–5631) were obtained from Agilent Technologies. A Qubit 3.0 Fluorometer and Qubit 1X dsDNA HS Assay kit (# Q33231) were obtained from Thermo Fisher Scientific. Quan-Plex Patient-like ctDNA Panel (AccuRef Diagnostics, # ARF-1003CT) was used as a reference cfDNA sample. CfDNA was extracted from human blood plasma using the following kits: QIAamp Circulating Nucleic Acid kit (Qiagen, # 55114), QIAamp MinElute ccfDNA kit (Qiagen, 55204), GenElute Plasma/Serum Cell-Free Circulating DNA Purification kit (MilliporeSigma, # DNB600-20RXN), MagMAX Cell-Free DNA Isolation kit (Thermo Fisher Scientific, # A29319), Quick-cfDNA Serum & Plasma kit (Zymo Research, # D4076), cfPure Cell Free DNA Extraction kit (BioChain, # K5011610), NucleoSnap DNA Plasma kit (Macherey-Nagel, # 740300), Plasma/Serum Cell-Free Circulating DNA Purification Kit (Norgen Biotek, # 55100), and Mag-Bind cfDNA kit (Omega Bio-tek, # M3298-01). Unless stated, the manufacturer's protocols and guidelines were followed.

cfDNA analysis

Analysis of cfDNA was performed according to the Cell-free DNA ScreenTape Quick Guide using the 4150 and 4200 TapeStation instruments with the TapeStation software revision 3.2 [1].

Results and discussion

cfDNA electropherogram profile

Cell-free DNA samples separated by automated electrophoresis with the Cell-free DNA ScreenTape assay display a prominent peak at approximately 170 bp followed by several smaller broad peaks (Figure 1). The prominent peak represents mononucleosome DNA fragments associated with one histone protein.

The less abundant DNA fragments are nucleosome multimers, often referred to as, for example, di- and tri-nucleosomes. These cfDNA multimers are larger in size, occurring at regular intervals from the mononucleosome (Figure 2). Some samples may also display HMW DNA above 700 bp. The Cell-free DNA ScreenTape assay evaluates cfDNA samples with a preset region from 50 to 700 bp, reflecting the percentage of sample that includes the cfDNA multimer fragments while excluding HMW DNA. While the pattern shown in Figure 2 with the mono- and dinucleosome fragments is typical of cfDNA, additional peaks representing larger nucleosome multimers may or may not be present, with the intensity of all the peaks varying greatly between samples. The diversity of each cfDNA electropherogram profile is primarily related to the sample's origin and the pre-analytical treatment of the sample.

CfDNA was extracted from human plasma using nine common commercially available cfDNA extraction kits and analyzed with the Cell-free DNA ScreenTape assay. The samples showed consistent electropherogram patterns, which demonstrated full compatibility of the Cell-free DNA ScreenTape assay with each of the tested extraction kits (data not shown).

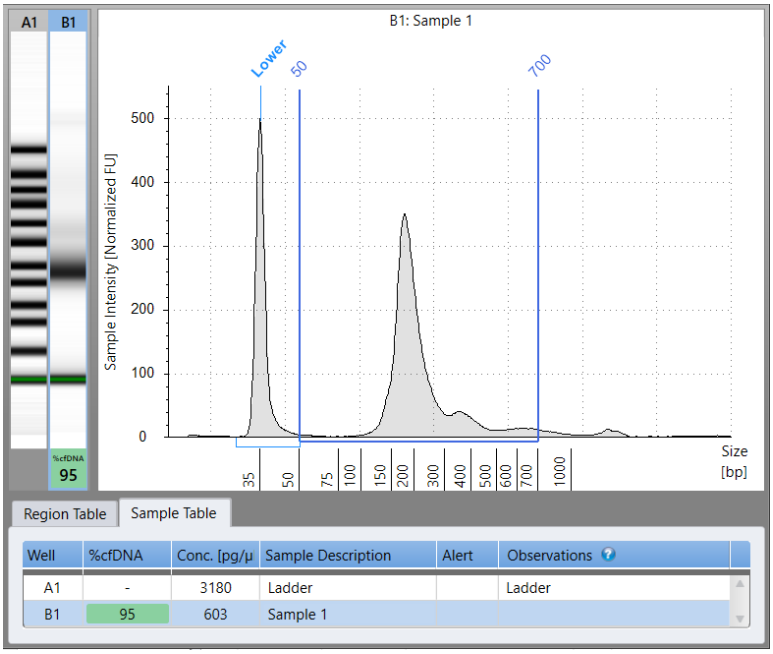

Figure 1. Typical electropherogram profile of a cell-free DNA sample analyzed with the Agilent Cell-free DNA ScreenTape assay. The prominent broad peak at approximately 170 bp represents the mononucleosome. Cell-free DNA is evaluated by the Agilent TapeStation analysis software with a preset region from 50 to 700 bp. The sample table displays the total DNA concentration and the cfDNA quality metric (%cfDNA).

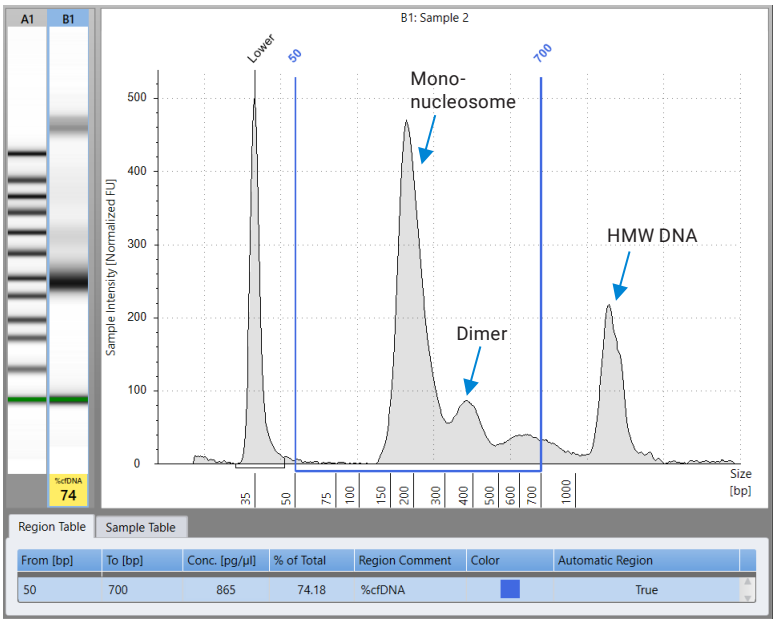

Figure 2. Characteristic profile of cfDNA analyzed with the Agilent Cell-free DNA ScreenTape assay. The electropherogram shows the typical peak of the mononucleosome, a smaller peak representing the dinucleosome, and high molecular weight (HMW) DNA. The mononucleosome and any multimers present are included in the %cfDNA region (50 to 700 bp), separating them from HMW DNA larger than 700 bp. The region table displays the concentration of cfDNA and percentage relative to the total DNA sample (%cfDNA).

Quality metric %cfDNA

During traditional NGS workflows, the presence of HMW DNA can negatively affect library yield and sequencing quality. The Cell-free DNA ScreenTape assay features a new quality metric, %cfDNA, reflecting the percentage of cfDNA subcomponents that are present in the preset region between 50 and 700 bp in relation to the total sample DNA. The %cfDNA metric allows for the user to evaluate sample quality and identifying whether a sample contains sufficient cfDNA for downstream processes. Figure 3 displays the electropherogram profiles of three samples with different %cfDNA values. The samples are composed of comparable amounts of cfDNA subcomponents but different HMW DNA quantity, resulting in different quality levels.

The accuracy of the %cfDNA metric was evaluated by adding sheared genomic DNA (gDNA) to a cfDNA reference sample. The unmixed reference sample had a quality of 85 %cfDNA. The sheared gDNA and reference sample were pooled at a variety of ratios to mimic samples covering a quality range from 10 %cfDNA to 85 %cfDNA. The mixed samples were analyzed in 24 replicates, with the Cell-free DNA ScreenTape assay and the resulting %cfDNA values plotted against the theoretical values determined from the mixing ratio (Figure 4). The results showed excellent correlation with an R^2 value of 0.993. A perfect correlation would result in an R^2 equal to 1. The average accuracy over all the data points was 3.8%, with an average precision of 3.3% CV.

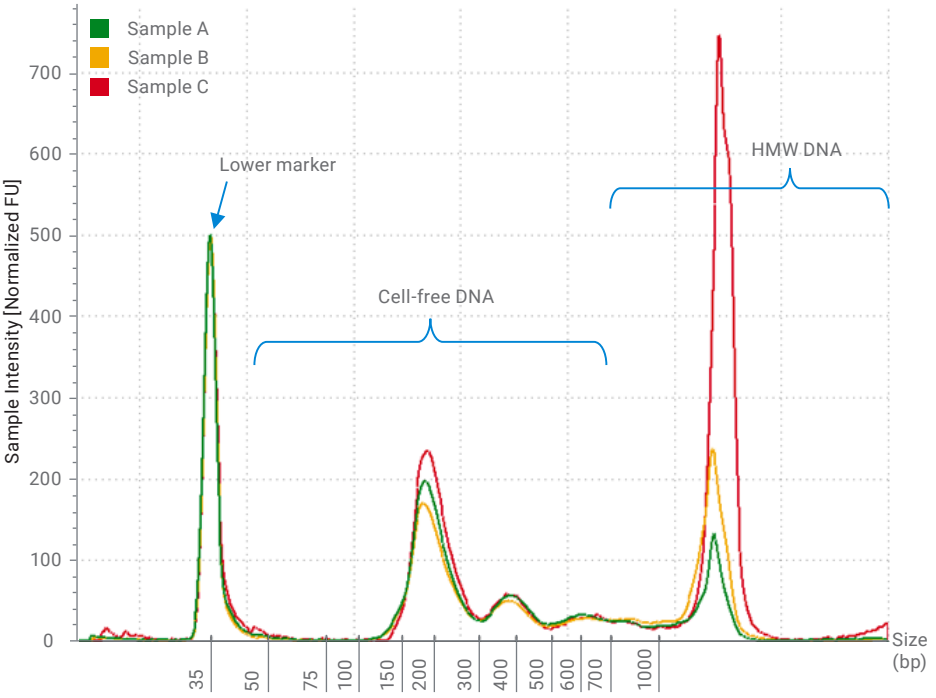

Figure 3. Electropherogram overlay of cfDNA samples with differing sample quality. Sample A (green, 72 %cfDNA) shows low abundance of HMW material > 700 bp, reflecting the highest quality of the sample set. Sample B (orange, 57 %cfDNA) has a lower quality because it contains a higher amount of HMW material. Sample C (red) displays the lowest quality value of 41 %cfDNA, due to a significant amount of HMW material.

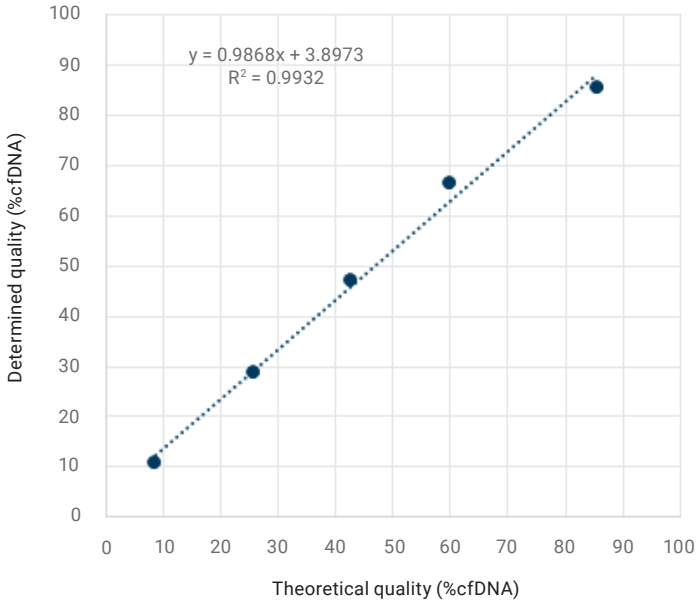

Figure 4. Accuracy of the %cfDNA metric evaluated using a reference sample pooled with HMW DNA. The theoretical values based on the pooling ratios are displayed on the X-axis, with a maximum of 85 %cfDNA represented by the unmixed reference sample. The prepared samples were analyzed with the Agilent Cell-free DNA ScreenTape assay, and the results of the %cfDNA value are presented on the Y-axis.

To demonstrate the concentration independence of the %cfDNA metric, a dilution series of a reference cfDNA sample (n=24), covering the %cfDNA functional range of the assay (Table 1), was analyzed (Figure 5). The average %cfDNA value was 85% ± 1.0 with a minimum value of 84% and maximum value of 87%. The %cfDNA results and the electropherogram profiles were consistent over the entire concentration range of the assay. In Figure 5, each concentration is overlaid with three different samples to demonstrate the consistency and precision of the Cell-free DNA ScreenTape assay. These results demonstrate that the quality metric %cfDNA provided by the Cell-free DNA ScreenTape assay is highly accurate and precise, and that the percentage is independent of the sample concentration.

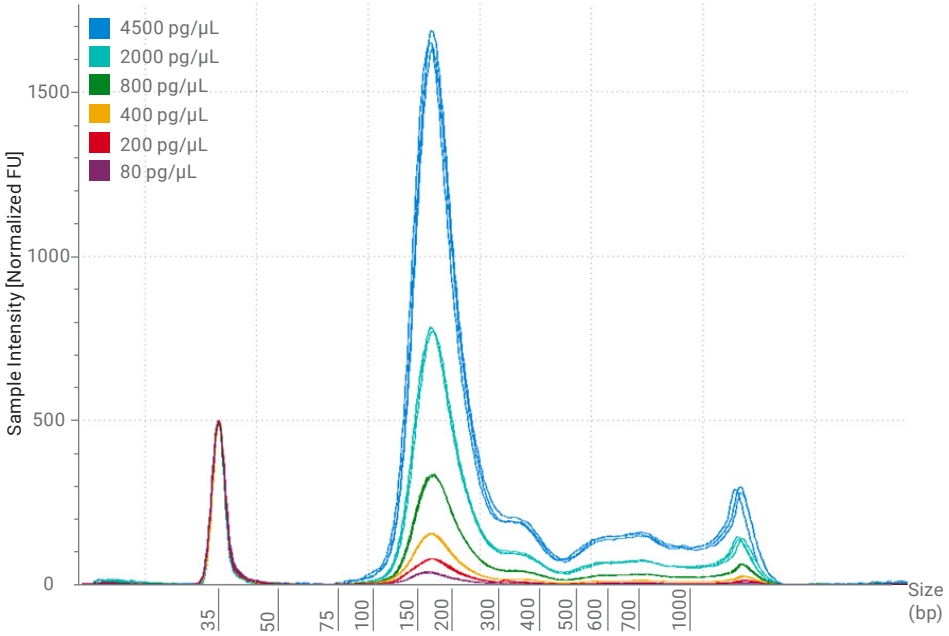

Figure 5. Dilution series of a reference cfDNA sample with an average quality value of 85 %cfDNA. Each concentration is overlaid with three different samples. The electropherogram profiles and the %cfDNA results are consistent over the entire assay concentration range.

Sensitivity

The sensitivity of the Cell-free DNA ScreenTape assay was evaluated with a 200 bp fragment and a cfDNA sample both diluted to 20 pg/µL, the detection limit of the assay (Table 1). Figure 6 shows the electropherogram overlay of both samples (n=5 replicates). The mononucleosome peak of the cfDNA sample shows a lower peak height compared to the fragment due to the broad peak shape. Nevertheless, both the fragment and the cfDNA sample are clearly detected with a signal-to-noise ratio (S/N) greater than 3, indicating that the sample peak is distinguishable from the baseline. These results validate the specified sensitivity of 20 pg/µL on the Cell-free DNA ScreenTape assay (Table 1) for cell-free DNA samples and for fragments.

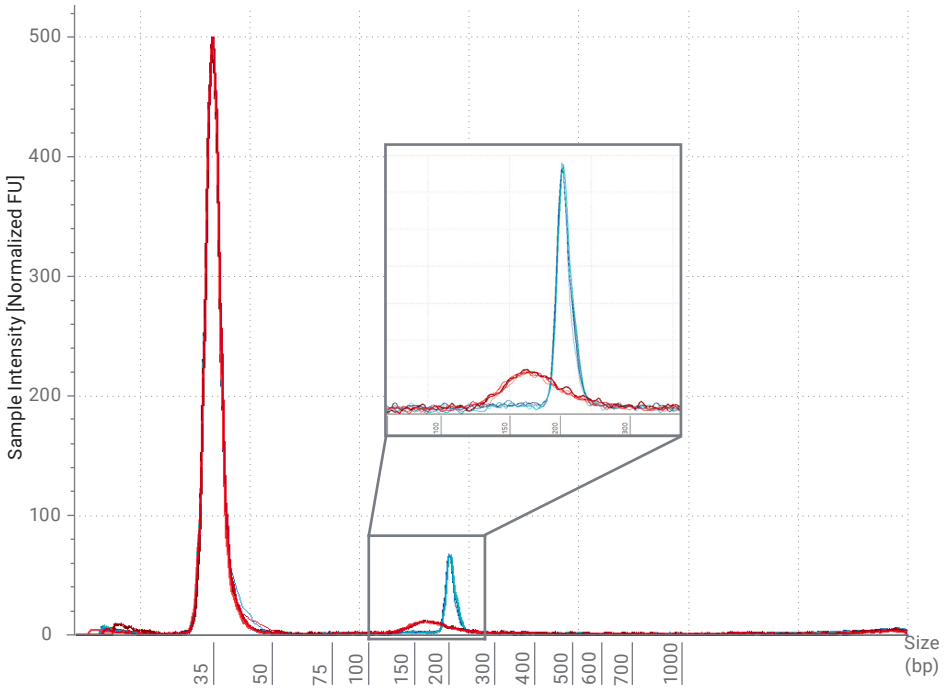

Figure 6. Electropherogram overlay (n=5) of a 200 bp fragment (blue) and a cfDNA sample (red), each with a concentration of 20 pg/µL analyzed with the Agilent Cell-free DNA ScreenTape assay. The cfDNA peak is clearly visible above the background signal.

Quantification

The Cell-free DNA ScreenTape assay separates cfDNA samples by size using automated electrophoresis. This enables determination of the total DNA concentration of the sample, as well as the quantification of the cfDNA portion apart from any HMW DNA present. The concentration of the cfDNA region and the total sample DNA are automatically evaluated by the TapeStation analysis software. The linear quantification range of the assay is 100 to 4,000 pg/µl total sample DNA as shown in Table 1.

To validate the specified linear quantitative range of the Cell-free DNA ScreenTape assay, a serial dilution of a reference cfDNA sample was quantified using three different 4150 and 4200 TapeStation instruments. The concentration resulting from the Cell-free DNA ScreenTape assay was similar to the concentration determined by Qubit (Figure 7) with an R^2 value of 0.998 for the 4150 TapeStation system and 0.997 for the 4200 TapeStation system.

The total DNA concentrations determined with the Cell-free DNA ScreenTape assay with both the 4150 and 4200 TapeStation systems displayed high correlation with the concentrations measured by Qubit. Both instruments displayed high quantitative accuracy within the specified limit of 20%.

The quantitative precision of the Cell-free DNA ScreenTape assay was determined using the same reference cfDNA dilution series. Figure 8 shows that the relative standard deviation was below 9% CV for each concentration analyzed with both the 4150 and 4200 TapeStation systems, which is well within the specified range of 15% CV (Table 1).

The Cell-free DNA ScreenTape assay showed excellent quantification performance in accuracy and precision, achieving consistent results with both the 4150 and 4200 TapeStation systems.

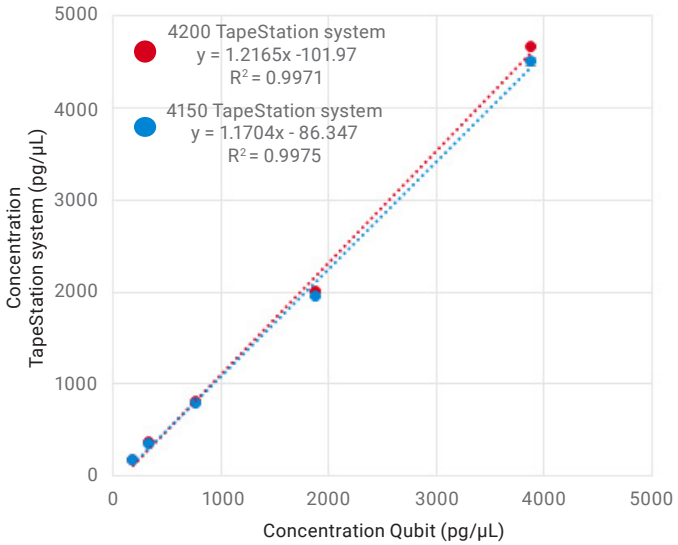

Figure 7. Quantification of a reference cfDNA sample in dilution series from 200 – 4000 pg/µL. Total sample DNA concentrations determined with the Cell-free DNA ScreenTape assay (n=24) on the 4150 and 4200 TapeStation systems were compared to DNA concentrations measured by Qubit (n=3).

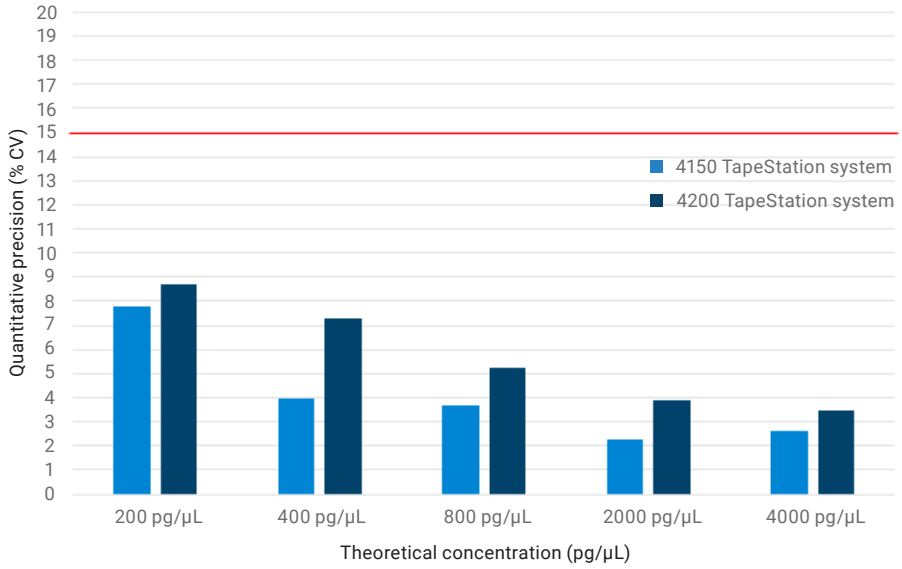

Figure 8. Quantitative precision of the Agilent Cell-free DNA ScreenTape assay (n=24) on the Agilent 4150 and 4200 TapeStation systems. The red line indicates the corresponding specified quantitative precision of the Cell-free DNA ScreenTape assay.

Sizing

The Cell-free DNA ScreenTape assay allows for sizing analysis of DNA from 50 to 800 bp. Larger DNA components of cfDNA samples are visible in the electropherogram and are marked as >800 bp in the electropherogram view.

To determine the sizing performance, the Cell-free DNA assay ladder was used as sample. The ladder consists of 10 individual DNA fragments within the sizing range of the assay. Figure 9 shows the sizing results from three different lots of ladder. Each DNA fragment size displayed a sizing accuracy below 4% deviation, which is within the specified sizing accuracy of ± 15% for the assay (Table 1).

The sizing precision was evaluated with the same ladder samples and is displayed as error bars in Figure 9. The determined sizing precision for the Cell-free DNA ScreenTape assay for each fragment was below 3% CV, which is well under the assay specification (10% CV) (Table 1). The sizing analyses obtained with the Cell-free DNA ScreenTape assay showed excellent accuracy and precision over the entire sizing range.

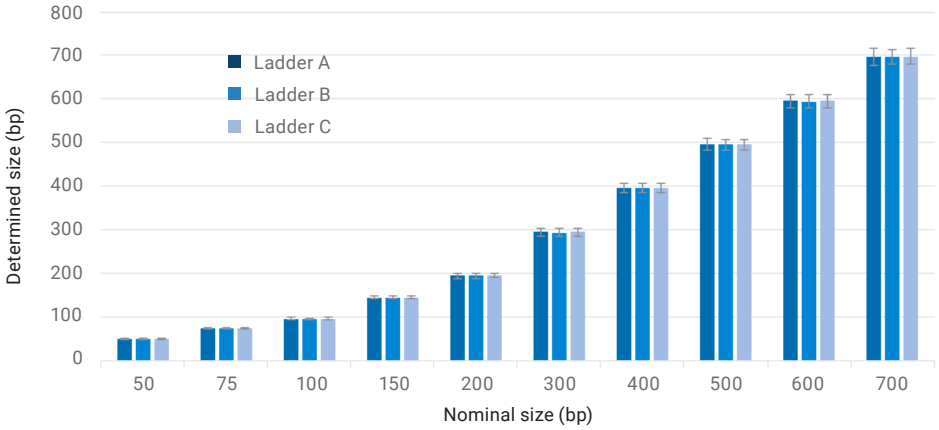

Figure 9. Sizing results for the 10 DNA ladder fragments of the Agilent Cell-free DNA ScreenTape assay from three different lots (n=175). Reported sizes were compared to the theoretical sizes.

Conclusion

This Technical Overview demonstrates excellent performance of the Cell-free DNA ScreenTape assay for accurate and precise quantification from 100 to 4,000 pg/µl and equivalent results compared to Qubit measurements. The assay delivered highly accurate sizing results in a range from 50 to 800 bp with remarkable precision. Moreover, automated region analysis enables separation and quantification of cfDNA subcomponents apart from high molecular weight material. The outstanding quality metric %cfDNA enables highly reliable and reproducible quality assessment of cfDNA samples independent of sample concentration. This makes the %cfDNA quality metric an effective tool for comparing the efficiency of pre-analytical treatments and defining quality thresholds for cfDNA as starting material for NGS library preparation. The compatibility of the assay with samples obtained by common cfDNA extraction kits demonstrates that the assay is appropriate for the majority of lab environments.

The 4150 and 4200 TapeStation systems delivered equivalent results, demonstrating seamless transition and full compatibility of both systems depending on throughput demand. The new low-throughput 4150 TapeStation system along with the Cell-free DNA ScreenTape assay offers the same ease-of-use combined with highly reliable quantification, sizing, and quality data for cfDNA samples at an economical budget.

References

1. Agilent Cell-free DNA ScreenTape Quick Guide for TapeStation Systems, *Agilent Technologies,* publication number G2991-90060, 2018.

www.agilent.com/genomics/tapestation

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