Application Note

Genomics

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Quality Control of Magnis SureSelect XT HS Workflow with Agilent Automated Electrophoresis Solutions

Authors

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Abstract

The Agilent 4150 TapeStation system performs reliable sample quality control (QC) through various steps of the SureSelect XT HS target enrichment and next-generation sequencing (NGS) library preparation workflow on the Agilent Magnis NGS Prep system. The workflow includes QC steps for the incoming samples, intermediate products during library preparation, and final libraries. The performance and applicability of the 4150 TapeStation system in qualification and quantification of libraries at different QC steps utilizing the TapeStation DNA ScreenTape assays is reviewed. The TapeStation DNA ScreenTape assays provided comparable results to the equivalent assays of the Agilent 2100 Bioanalyzer and 5200 Fragment Analyzer systems. Final library QC metrics were correlated to the outcome of QC metrics from sequencing data.

Introduction

Detection and characterization of somatic genomic variations in cancer specimens is routine in translational research, as it facilitates diagnostic and individualized therapy selection towards cancer treatment. Targeted panel and exome sequencing are powerful NGS tools that can identify such variants from cancer samples. Due to their growing popularity in diagnostic laboratories, NGS technologies and methods involving such approaches have developed over the years. However, the process of NGS sample preparation is still very complex, as manual protocols are time consuming, requiring considerable expertise, optimization, and validation for diverse applications.

The Agilent Magnis NGS Prep system is an automated NGS library preparation solution that addresses such challenges. It leverages the industry-leading target enrichment solution, the Agilent SureSelect XT HS system. With a predefined SureSelect XT HS protocol and prepackaged reagents, the automated liquid handling system can be set up with minimal experience, in a very short time, for preparing NGS libraries from eight different samples in approximately nine hours. The Magnis NGS Prep system produces high-quality and highly reproducible target-enriched NGS libraries with starting material of fragmented genomic DNA (gDNA) purified from blood, fresh frozen tissues, or degraded FFPE samples. The steps of library preparation and target enrichment are executed within the Magnis system without user intervention (Figure 1). Despite automation and reproducibility, it is beneficial to perform QC steps and quantification on the starting material, the materials derived from intermediate steps (optional), and the final library to ensure reliability and overall success of the sequencing data (Figure 1).

Agilent has several automated electrophoresis platforms for sample QC in the NGS workflow. The Agilent 4150 TapeStation system is an ideal platform for quality and quantity assessment of samples from critical QC steps of the Magnis system workflow. It is a low-throughput system for 1 to 16 samples, offering short preparation time and rapid analysis of 1 to 2 minutes per sample.

This application note details the quality control of NGS library preparation following the Magnis SureSelect XT HS target enrichment workflow on the 4150 TapeStation system with the Genomic DNA ScreenTape, D1000 ScreenTape, and High Sensitivity D1000 ScreenTape assays. It also compares the capabilities and performance of quality assessment for DNA assays on similar low-throughput electrophoresis platforms offered by Agilent, such as the 2100 Bioanalyzer and 5200 Fragment Analyzer systems.

Experimental

Materials

Qubit BR dsDNA HS Assay Kit (p/n Q32850) and 1X Low TE buffer (p/n 12090-015) were from Thermo Fisher Scientific Inc. The Covaris E220 instrument and the 96 microTUBE plate (p/n 520045) from Covaris (Woburn, MA, USA) were used. HapMap genomic DNA (gDNA) sample (p/n NA18507) was obtained from Coriell Cell Repositories. Reference standards representing DNA with quality compromised by formalin fixation (p/n HD798, HD799, and HD803) were obtained from Horizon Discovery. gDNA from Horizon FFPE tissue sections was isolated using Qiagen QIAamp DNA FFPE Tissue Kit (p/n 56404) and Qiagen Deparaffinization Solution (p/n 19093) according to the manufacturer's protocol.

The following were from Agilent Technologies (Santa Clara, USA):

Agilent Magnis NGS Prep system (p/n G9710AA)

Agilent 4150 TapeStation system (p/n G2992AA)

Agilent 2100 Bioanalyzer system (p/n G2939BA)

Agilent 5200 Fragment Analyzer system (p/n M5310AA)

Agilent Genomic DNA ScreenTape (p/n 5067-5365)

Agilent Genomic DNA reagents (p/n 5067-5366)

Agilent D1000 ScreenTape (p/n 5067-5582)

Agilent D1000 reagents (p/n 5067-5583)

Agilent High Sensitivity D1000 ScreenTape (p/n 5067-5584)

Agilent High Sensitivity D1000 reagents (p/n 5067-5585)

Agilent High Sensitivity DNA kit (p/n 5067-4626)

Agilent DNA 1000 kit (p/n 5067-1504)

Agilent NGS Fragment kit (1-6000 bp) (p/n DNF-473-0500)

Agilent HS NGS Fragment kit (1-6000 bp) (p/n DNF-474-0500)

Agilent NGS FFPE QC kit (p/n G9700A)

Agilent AriaMx Real-Time PCR system (p/n G8830A)

Magnis SureSelect XT HS 0.5-2.9Mb Bait plate, 96 Reactions (HD200 small, design ID in SureCall: 3058371)

Library preparation

Library preparation was done following sample preparation guidelines in Magnis SureSelect XT HS Target Enrichment manual[1]. The gDNA samples were quantified using Qubit BR dsDNA assay or Agilent NGS FFPE QC kit as per manufacturer's instructions for the instrument and assay kit. After quality assessment, 10 ng of each gDNA sample was diluted using 1X Low TE buffer to a final volume of 50 µL. DNA samples were fragmented using Covaris E220 and the sheared gDNA samples were directly transferred to a Magnis sample input strip and loaded onto the Magnis system along with preloaded reagent plates, probe strips for ClearSeq Comprehensive Cancer Panel, and labware for subsequent steps of library preparation following the protocol as detailed in the Magnis SureSelect XT HS Target Enrichment Manual[1]. During run setup, the option "Aliquot sample for QC" was selected from the "Enter Run Info" screen to enable collection of pre-capture library samples.

DNA analysis

The 4150 TapeStation with the Genomic DNA ScreenTape assay was used to assess the integrity of input gDNA. Post-shear and post-capture DNA libraries were analyzed with the 4150 TapeStation High Sensitivity D1000 ScreenTape assay, 2100 Bioanalyzer High Sensitivity DNA assay, and 5200 Fragment Analyzer HS NGS Fragment assay. Pre-capture DNA libraries were analyzed with the 4150 TapeStation D1000 ScreenTape assay, 2100 Bioanalyzer DNA 1000 assay, and 5200 Fragment Analyzer NGS Fragment assay. Quantity and size of pre- and post-capture DNA libraries were measured using the region functionality. Unless stated otherwise, the manufacturer's protocols and guidelines were followed.

Sequencing

The libraries were normalized to 10 nM, equimolar pooled, transferred to a sequencing flow cell, loaded onto HiSeq2500, and sequenced at 4000x. For normalization, the region molarity of the finished libraries determined by the 4150 TapeStation system was used. Sequencing metrics including on-target percentage and read depth were analyzed with Agilent internal pipeline and compared between Magnis system and manual library preparation. SureCall software was used to analyze SureSelect XT HS libraries for determining allele frequency and call rates.

Results and Discussion

The Magnis SureSelect XT HS target enrichment workflow includes several QC points for overall success in library preparation: input DNA, post-shear, and post-capture library, as illustrated in Figure 1. There is also an optional step for collection and QC of pre-capture library at the end of the Magnis system workflow for customers who intend to use or analyze samples from this step. The samples were prepared in replicates of eight from high-quality HapMap DNA or FFPE DNA at varying levels of degradation on the Magnis system.

Quantity and integrity check on input gDNA

To determine if the samples are suitable for library preparation, QC was done on all four sample types. The Magnis SureSelect XT HS assay requires input of 10, 50, 100, or 200 ng DNA suspended in a total volume of 50 µL 1X Low TE buffer. It is important that all samples in the same run contain equal amounts of starting DNA. High-quality input DNA from HapMap sample NA18507 was quantified by the Qubit system.

It is recommended that lower quality samples are analyzed with the qPCR-based assay and the Agilent NGS FFPE QC kit[2]. Quantification of FFPE samples relies on the qualification score supplied by the NGS FFPE QC kit. The results of this assay provide a ΔΔCq DNA integrity score and precise quantity of amplifiable DNA in the samples. Users may follow Table 1 for quantification guidelines based on the ΔΔCq integrity score. For samples with a ΔΔCq score of ≤1, such as mildly degraded FFPE DNA HD798, a Qubit-based gDNA quantification was used to determine the amount of input DNA needed for the protocol.

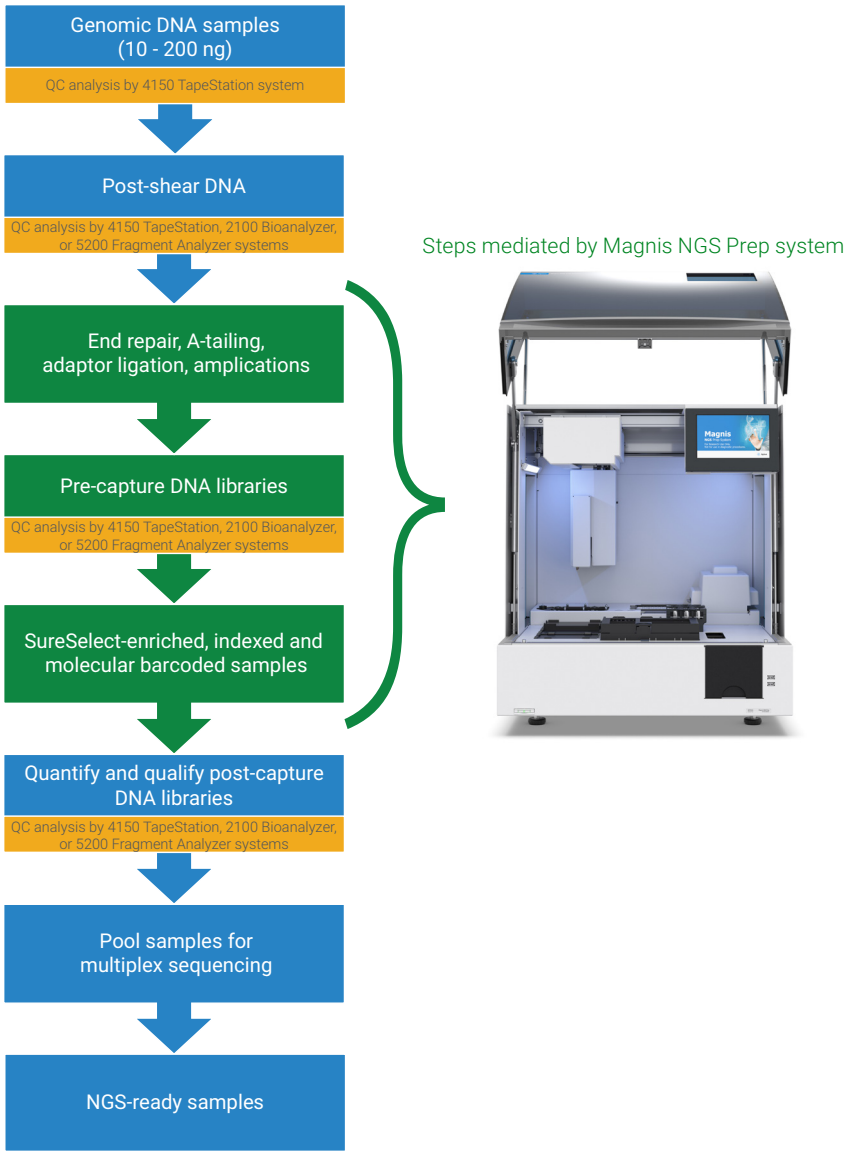

Figure 1. Workflow overview of Agilent SureSelect XT HS enrichment using the Magnis NGS Prep system with recommended QC checkpoints using the 4150 TapeStation, 2100 Bioanalyzer, or 5200 Fragment Analyzer systems. Magnis system mediated steps are highlighted in green.

Table 1. SureSelect XT HS gDNA input modifications based on ΔΔCq DNA integrity score.

Protocol Parameter	Non-FFPE Samples	FFPE Samples	
		ΔΔCq≤1*	ΔΔCq>1
DNA Input for Library Preparation	10 ng, 50 ng, 100 ng or 200 ng DNA, based on Qubit assay	10 ng, 50 ng, 100 ng or 200 ng DNA, based on Qubit assay	10 ng, 50 ng, 100 ng or 200 ng of amplifiable DNA, based on qPCR quantification

**FFPE samples with DDCq≤1 should be treated like non-FFPE samples for DNA input amount determinations. For sample of this type, make sure to use the DNA concentration determined by Qubit assay, instead of concentration determined by qPCR, to calculate the volume required for 10 to 200 ng DNA.*

For moderately and severely degraded FFPE samples HD799 and HD803, where the ΔΔCq score was >1, qPCR-based quantitation of amplifiable DNA reported by the results of the NGS FFPE QC kit was used[2]. The Magnis system allows for varying amounts of input DNA, and whenever possible the use of higher concentrations is advisable. In order to demonstrate the efficacy of the Magnis system in NGS library preparation, scarce or low-yield samples were used. The least permissible amount of 10 ng suspended in 50 µL of 1X Low TE was chosen for all four sample types.

An alternate method of gDNA integrity assessment utilizes the Genomic DNA ScreenTape assay on the 4150 TapeStation system. This assay gives a quality score or DNA integrity number (DIN) for samples analyzed (Figure 2). The DIN algorithm is a feature of the TapeStation analysis software that provides a numerical assessment of the DNA sample by assigning a numerical score for DIN from 1 to 10[3]. While a high DIN is indicative of good quality and intact gDNA, a low DIN is suggestive of a strongly degraded gDNA sample. The DIN score is particularly essential for FFPE samples as they are often degraded. The score is used to optimize protocol for the fragmentation step and in determining the amount of input DNA, as measured by the Qubit, to be used for library preparation. Table 2 explains the quantification guidelines based on the DIN score from TapeStation data. A high-quality DNA or mildly degraded FFPE sample with a DIN score >8, such as lane B1 in Figure 2A, will allow input as low as 10 ng, for the Magnis SureSelect XT HS protocol.

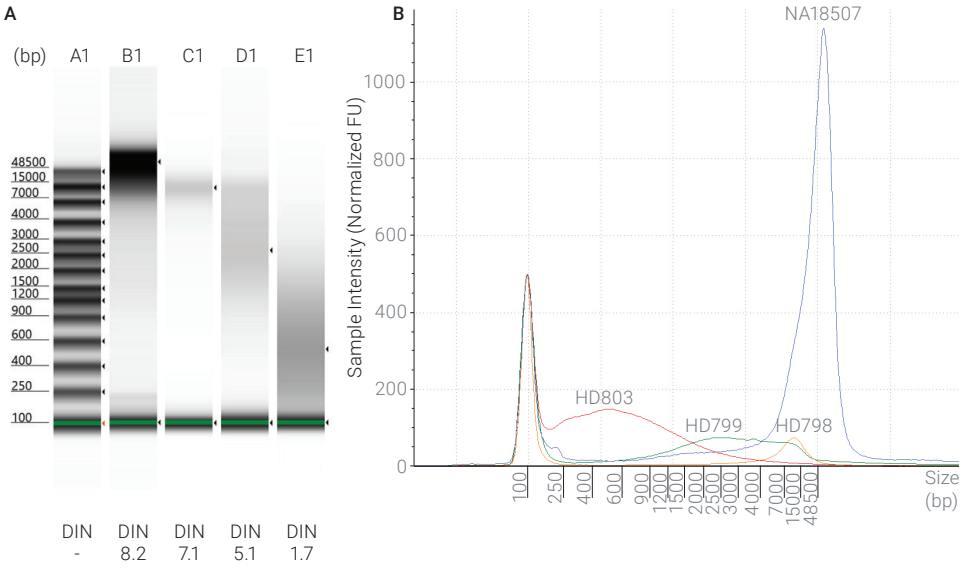

Figure 2. Assessment of gDNA integrity of input DNA samples using Genomic DNA ScreenTape assay on the 4150 TapeStation system. (A) Gel image is shown with DIN, indicating the gDNA integrity of each sample. (B) Electropherogram overlay of input DNA from different sources. A1: genomic DNA ladder; B1: high-quality HapMap genomic DNA NA18507; C1: mildly degraded FFPE sample HD798; D1: moderately degraded FFPE sample HD799; E1: highly degraded FFPE sample HD803.

Table 2. SureSelect XT HS DNA input modification based on DNA Integrity Number (DIN) score.

Protocol Parameter	Non-FFPE Samples	FFPE Samples		
		DIN >8*	DIN 3-8	DIN <3
DNA Input for Library Preparation	10 ng, 50 ng, 100 ng or 200 ng DNA, based on Qubit assay	10 ng, 50 ng, 100 ng or 200 ng DNA, based on Qubit assay	Use 50 ng, 100 ng or 200 ng DNA (use the maximum amount of DNA available, up to 200 ng). Quantity by Qubit assay to determine volume required for 50 ng, 100 ng or 200 ng input.	Use 100 ng or 200 ng DNA (use the maximum amount of DNA available, up to 200 ng). Quantity by Qubit assay to determine volume required for 100 ng or 200 ng input.

** FFPE samples with DIN>8 should be treated like non-FFPE samples for DNA input amount determinations.*

Unlike the qPCR-based method described above, for samples found to be moderately and severely degraded by the 4150 TapeStation with a DIN below 8, input quantity measured as low as 10 ng by Qubit system cannot be used with full confidence in the load. If the DIN is between 3 and 8, then it is recommended to use higher amounts of inputs, e.g., 50, 100, or 200 ng. For severely degraded FFPE samples with DIN <3 it is best to use 100 or 200 ng of input. For all instances where a sample has a DIN below 8, it is best to use the highest amount of input (200 ng) available for best possible results.

Characterization of sheared DNA

The first step of the SureSelect XT HS protocol is fragmentation of gDNA by shearing with a Covaris Ultrasonicator. The intact gDNA should be in 50 µL volume of 1X Low TE buffer, as shearing samples diluted in water or any other solvents can result in loss of complexity or yield of the library. Unlike FFPE samples, high-quality intact gDNA requires two rounds of shearing[1]. Sheared gDNA analyzed on the 4150 TapeStation system with the High Sensitivity D1000 ScreenTape assay is expected to give a single smear distribution with a median range of 150 to 200 bp (Figure 3). Assessment of the size and uniformity of sheared DNA at this step is useful for establishing size comparison in subsequent steps of the workflow. Uneven shearing or larger than expected library size is a result of suboptimal shearing commonly associated with bubbles in the microTUBE filament of the Covaris system, inadequate sample volume, or DNA suspended with an incorrect buffer. Adherence to the details of shearing protocol, including spinning, vortexing, and using the correct amount/volume of input DNA, can eliminate such aberrations.

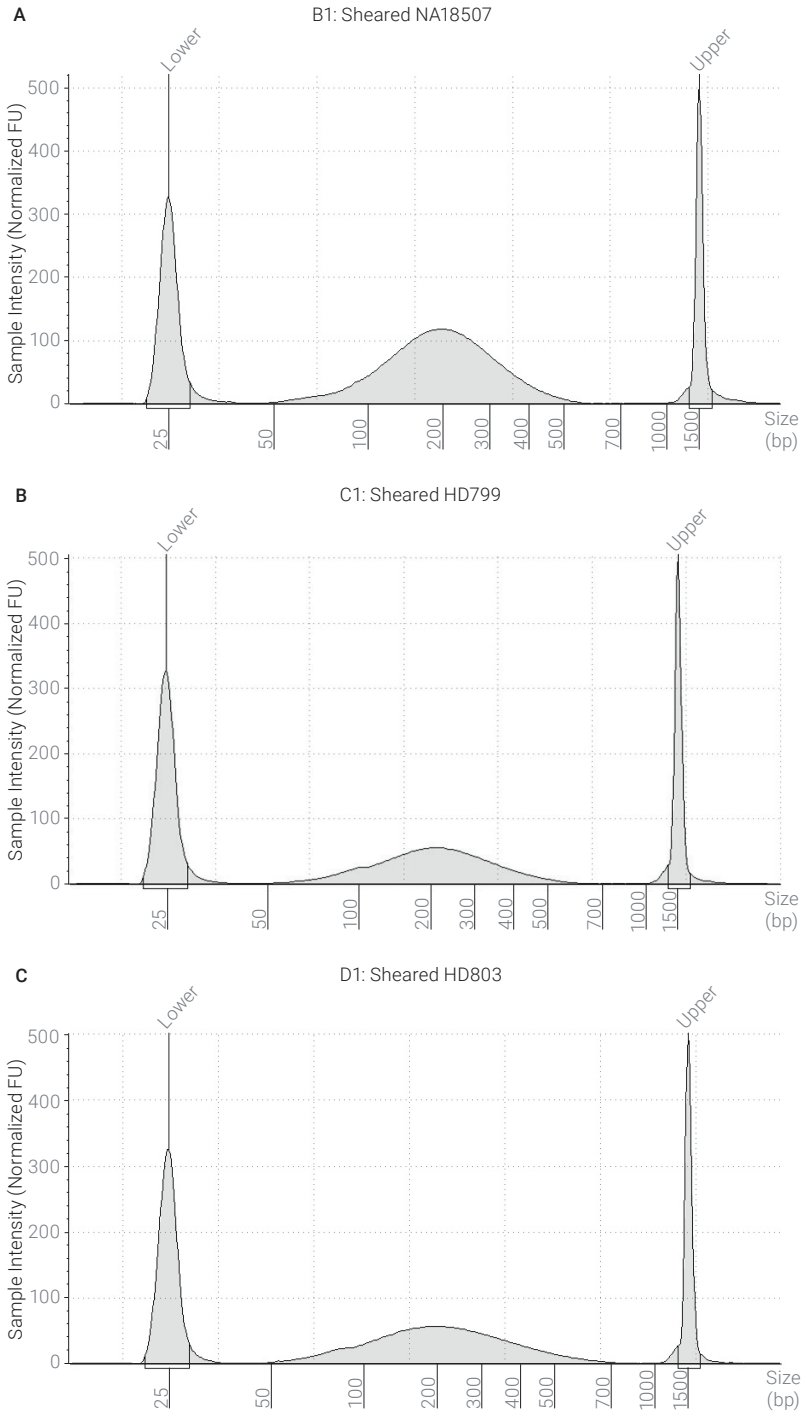

Figure 3. Electropherogram pattern of sheared DNA analyzed with the High Sensitivity D1000 ScreenTape assay on the 4150 TapeStation system, showing DNA fragment size peak between 150 to 200 bp for (A) high-quality HapMap DNA input NA18507, (B) moderately degraded FFPE sample HD799, and (C) highly degraded FFPE sample HD803.

Qualification of pre-capture libraries

The sheared samples are transferred to Magnis sample input strips and the subsequent pre-capture library preparation steps of end repair, A-tailing, molecular barcoded adaptor ligation, amplification, and magnetic bead purification take place automatically within the Magnis system. However, the pre-capture library samples from this step can only be analyzed at the end of a completed run along with the final target enriched NGS library. Hence, the pre-capture library is an optional QC step that users can choose during run setup. Pre-capture library QC assesses the size and concentration of the libraries, which is essential for customers who would like to sequence it at this stage. It also helps in identifying and troubleshooting sample quality, preparation or reagent-related issues leading to failed sequencing of the final library.

To include this option, the "Aliquot sample for QC" check box was selected from the "Enter Run Info" screen, enabling the Magnis system to collect a 3 µL aliquot of pre-capture library before moving to the next step. Users are required to fully air dry the collected aliquot and suspend it in 6 µL of water per sample for sizing and quantification on an 4150 TapeStation system with the D1000 ScreenTape assay. A size shift from adaptor ligation is expected at this step, and the average size of pre-capture library ranges between 300 and 400 bp for libraries prepared from high-quality intact DNA and 200 to 400 bp for libraries prepared from FFPE DNA (Figure 4). It is not unusual to see a low molecular weight peak in addition to the expected pre-capture library smear due to the presence of adaptor dimers, especially in the FFPE sample set.

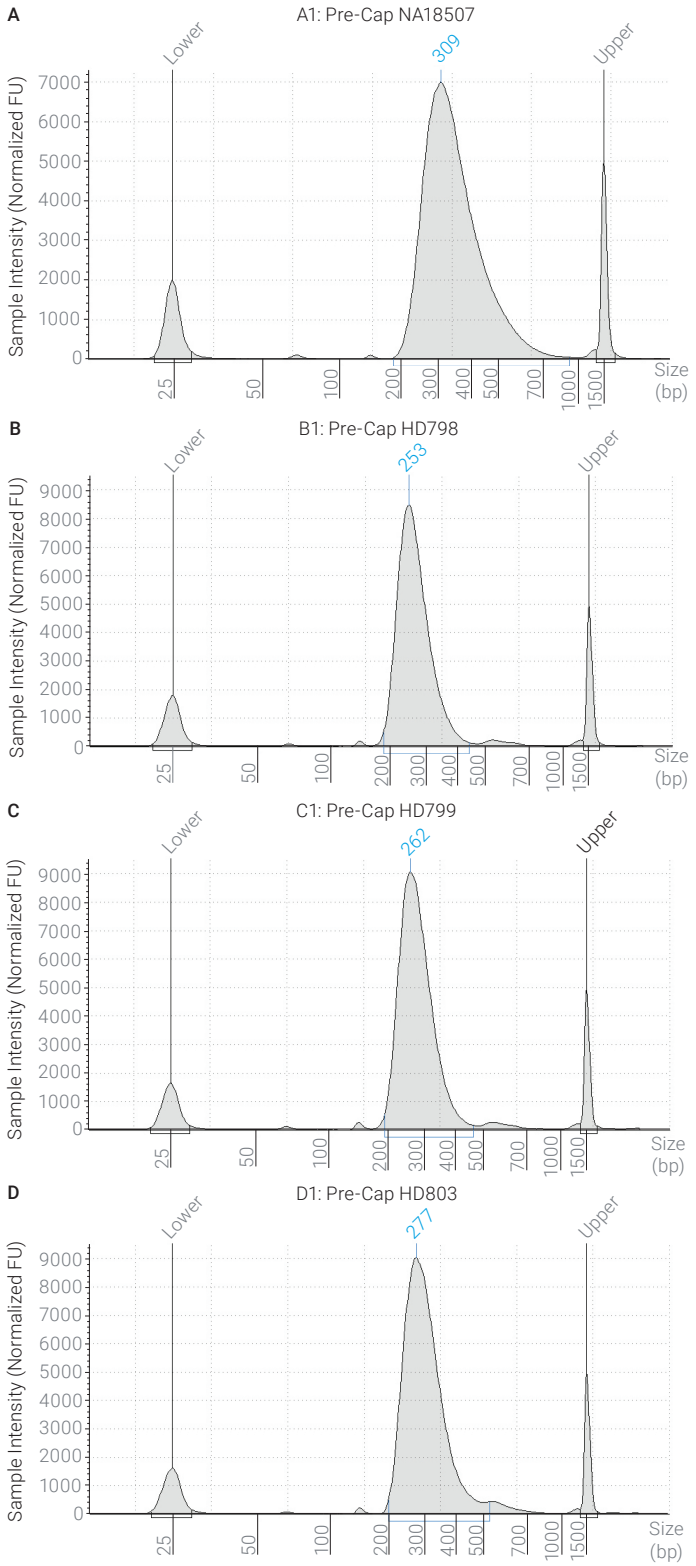

Figure 4. Electropherogram pattern of Magnis SureSelect XT HS pre-capture DNA library separated with the D1000 ScreenTape assay on the 4150 TapeStation system, showing the expected maximum peak size between 300 and 400 bp for (A) high-quality HapMap DNA input NA18507 and between 200 and 400 bp for (B) mildly degraded FFPE sample HD798, (C) moderately degraded FFPE sample HD799, and (D) highly degraded FFPE sample HD803.

However, the presence of adaptor dimers was not seen to impact the following steps of target enrichment, or the yield and quality of the post-capture library. Concentration of the pre-capture library suspended in
6 µL of water ranged from 50 to 75 ng/µL (expected 30 to 100 ng/µL), depending on the quality of the input DNA and number of cycles applied for PCR amplification. To consider the dilutions and sample adjustments in preparation of the pre-capture library, the overall yield is calculated as the amount of DNA in 1 µL of reconstituted QC sample times 36. One of the common reasons for no or low pre-capture yield is inadequate resuspension or loss of dried-down material from the collected aliquot.

Final library QC

The final product of this workflow is the post-capture library resulting from hybridization of adaptor-ligated libraries with target enrichment probes, followed by capture and amplification on the Magnis system. Libraries should be pooled on an equimolar basis for multiplexed sequencing. Hence, QC analysis to assess the size distribution and yield is critical for sequencing success. The final libraries from the four different sample types were evaluated for size distribution and molarity with the High Sensitivity D1000 assay on the 4150 TapeStation system. Samples were seen to have an evenly distributed broad smear with an expected average size between 200 and 400 bp (Figure 5). The molarity was assessed for the selected smear range for each sample. Library samples to be pooled were each diluted to 10 nM and equal volumes were combined for the final sequencing pool.
An individual sample with low yield at this step may indicate suboptimal amplification, requiring users to increase the PCR cycle number by one or two cycles when repeating the library preparation and target enrichment.

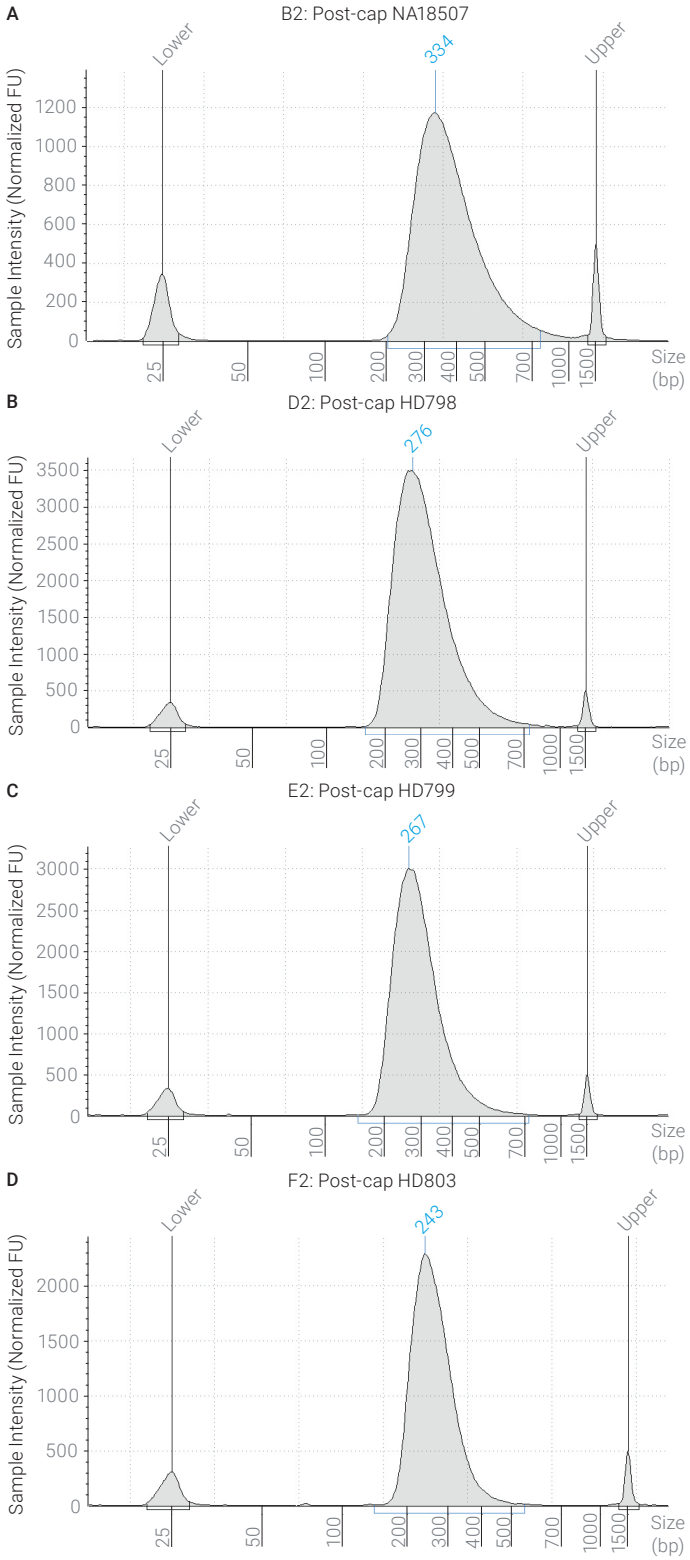

Figure 5. Electropherogram pattern of Magnis SureSelect XT HS post-capture DNA library separated with the High Sensitivity D1000 ScreenTape assay on the 4150 TapeStation system, showing the expected maximum peak size between 200 and 400 bp for (A) high-quality HapMap DNA input NA18507, (B) mildly degraded FFPE sample HD798, (C) moderately degraded FFPE sample HD799, and (D) highly degraded FFPE sample HD803.

Additionally, it could also be from input DNA failing to meet the quality and concentration requirements, hence the emphasis on input DNA QC, as discussed earlier in this note. A very low yield, below 2 nM or a complete dropout, is most likely from setup issues like labware out of position or from operating the instrument in an environment outside the specified humidity range of 30 to 70%.

Comparison of QC instruments

In addition to the 4150 TapeStation system, Agilent has two other low-throughput, automated electrophoresis platforms, the 2100 Bioanalyzer and the 5200 Fragment Analyzer systems. These also gave comparable and accurate QC data from various stages of the Magnis system library preparation workflow (Figure 6). The 2100 Bioanalyzer and the 5200 Fragment Analyzer systems can be used to qualify sheared DNA, pre-capture and post-capture libraries with sizing results equivalent to the 4150 TapeStation system.

Sequencing

Table 3 summarizes and compares the sequencing QC metrics of SureSelect XT HS libraries prepared mechanically by the Magnis system versus manually processed libraries with an identical set of input samples and probes. The automated Magnis system provided similar sequencing results to manually processed libraries for high quality down to severely degraded FFPE samples. Successful sequencing results were obtained with the minimal input concentration for all sample types, but to achieve consistent and reliable sequencing results with degraded samples such as FFPE, higher concentrations of 100 to 200 ng/µL is recommended. The Magnis system can reliably be used for high throughput library prep workflows regardless of the type of sample.

Figure 6. Average sizes of Magnis SureSelect XT HS post-shear, pre-capture, and post-capture libraries determined by the 4150 TapeStation, 2100 Bioanalyzer, and 5200 Fragment Analyzer systems. Post-shear DNA and post-capture library were analyzed with the High Sensitivity D1000 ScreenTape assay, High Sensitivity DNA assay, and HS NGS Fragment assay on a 4150 TapeStation, 2100 Bioanalyzer, and 5200 Fragment Analyzer systems, respectively. Pre-capture library was analyzed with a D1000 ScreenTape assay, DNA 1000 assay, and NGS Fragment assay on a 4150 TapeStation, 2100 Bioanalyzer, and 5200 Fragment Analyzer systems, respectively.

Table 3. Comparison of SureSelect XT HS library sequencing QC metrics for libraries prepared using the Magnis NGS Prep system versus manually prepared samples. Both protocols used 10 ng input DNA, target-enriched with CCP probes. Averaged sequencing metrics at 100X raw depth for 8 samples of high-quality HapMap DNA input NA18507 and 10 samples each for mildly degraded FFPE sample HD798, moderately degraded FFPE sample HD799, and severely degraded FFPE sample HD803.

NA18507	Magnis (10 ng)	Manual (10 ng)	HD798	Magnis (10 ng)	Manual (10 ng)
% Duplicates	7	7	% Duplicates	12	12
% Reads in Targeted Regions	67	69	% Reads in Targeted Regions	69	70
At Least 20x Reads	81	82	At Least 20x Reads	91	91
Complexity (Library Size)	1,823,778	1,947,407	Complexity (Library Size)	158,706	103,661
HD799	**Magnis (10 ng)**	**Manual (10 ng)**	**HD803**	**Magnis (10 ng)**	**Manual (10 ng)**
% Duplicates	17	24	% Duplicates	27	31
% Reads in Targeted Regions	70	71	% Reads in Targeted Regions	72	69
At Least 20x Reads	90	89	At Least 20x Reads	81	76
Complexity (Library Size)	95,512	90,306	Complexity (Library Size)	70,198	51,526

Conclusions

The automated Agilent Magnis NGS Prep system is a reliable and convenient tool for target-enriched NGS library preparation, made even more robust by use of the Agilent automated electrophoresis platforms for QC steps throughout the workflow. The QC attained by ready-to-use ScreenTape devices on the Agilent 4150 TapeStation system identifies properly prepared library samples with minimal hands-on time. QC is critical for properly classifying the integrity of input samples and sizing of the final libraries, all necessary for consistent and reliable sequencing. The automated Magnis system provided similar sequencing results to manual library preparation, demonstrating its reliability for high throughput workflows. Agilent automated electrophoresis platforms, used in conjunction with the Magnis system, enables optimization of the sequencing library preparation process from the beginning to end, ensuring high-quality sequencing results.

References

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