BECKMAN COULTER
Life Sciences

Automation of Illumina TruSight Oncology 500 DNA Only on the Biomek NGeniuS Next Generation Library Prep System

Abstract

As genome sequencing and data analysis methods become more accessible, more laboratories are exploring NGS (Next-Generation Sequencing) as a research tool, particularly in the field of cancer research where the elucidation of driver mutations allows researchers to develop new therapies against various types of cancers. In this paper, we detail an automated process for the Illumina TruSight Oncology 500 DNA Only library preparation kit. The Biomek NGeniuS Library Prep System offers the laboratory optional settings to optimize a demonstrated application that will process between 4 and 16 samples from start to finish, with minimal interaction from the user. Sequencing results from library preparation indicate that over 98% of variants at 5% variant allele frequency (VAF) and higher can be successfully identified from formalin-compromised samples.

Introduction

The completion of the Human Genome Project in 2003 heralded a new era in biomedical research. Not only did researchers have more knowledge about the human genome than ever before, they also had mature computer programs to analyze data and assist in discovering the interactions between changes in genetic information and disease states. Despite these advances, the cost to sequence was still prohibitive to obtain degrees of coverage required for clinicians to be certain of linkages between genotype and disease phenotype. In 2005, massively parallel sequencing (or Next-Generation Sequencing, referred to as "NGS") was introduced to the scientific community, allowing for much larger sequencing datasets to be generated. The depth of sequencing coverage provided by NGS allows research to investigate rare variants that can be drivers of many types of cancers.

The Illumina TruSight Oncology 500 DNA Only assay is a comprehensive genomic profiling (CGP) NGS research-use-only assay targeting the full coding regions of 523 genes implicated in the pathogenesis of solid tumors. Employing an enrichment-based library preparation strategy for use with formalin-fixed, paraffin-embedded (FFPE) samples, TruSight Oncology 500 DNA Only can detect small variants (single nucleotide variants [SNVs], multinucleotide variants [MNVs], and indels), and amplifications in a single sequencing run. TruSight Oncology 500 DNA Only also detects immunotherapy biomarkers for tumor mutational burden (TMB) and microsatellite instability (MSI).

The Illumina TruSight Oncology 500 DNA Only assay automated on the Biomek NGeniuS Next Generation Library Prep System provides high-quality, reproducible libraries. General workflow steps for this kit are outlined in **Figure 1**. Briefly, normalized DNA (either genomic DNA or DNA derived from formalin fixed paraffin embedded (FFPE)) is sheared by the operator off instrument using a Covaris instrument. The automation workflow supports the same recommended input ranges of DNA (40 ng to 120 ng) as the manual protocol. The operator may wish to adjust either the Covaris shearing settings or the input mass of DNA based on sample quality. Please refer to the TruSight Oncology 500 Reference Guide for additional recommendations on sample input and quality. Following shearing, the DNA samples are then loaded onto the Biomek NGeniuS Next Generation Library Prep System, and the first series of operations (end repair, A-tailing, adapter ligation, and index PCR) are performed, after which the automation workflow is halted to allow recovery of the amplified libraries. The automation method is then restarted for the second series of operations (including first hybridization, first capture, second hybridization, second capture, enrichment PCR and enrichment PCR cleanup), after which the automation workflow is halted to allow for an optional quality control step. The third and final operation, bead-based library normalization, is then performed on the Biomek NGeniuS Next Generation Library Prep System, resulting in normalized libraries ready to be sequenced. This application does not support either the combined RNA/DNA workflow or the HRD specific workflow described in the TruSight Oncology 500 Reference Guide. The application is capable of processing batches of any size between 4 and 16 samples and is capable of processing a 16-sample batch in sufficient time for the libraries to be placed on the Illumina sequencer at the end of the second day of the batch run, with only three user interactions during the course of the batch run.

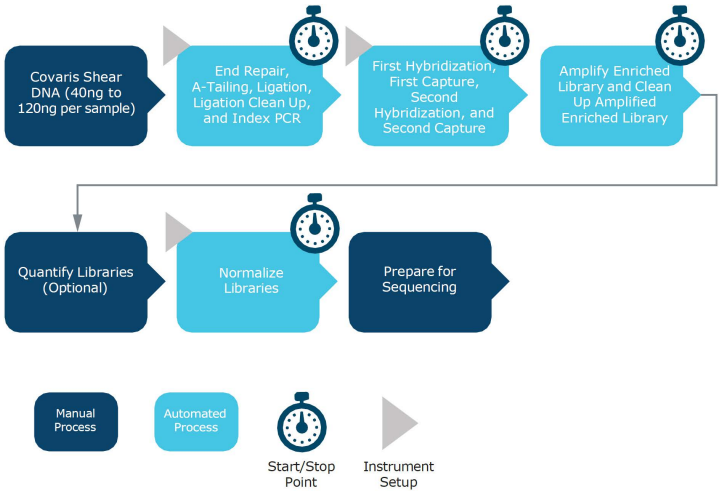

Figure 1. Illumina TruSight Oncology 500 DNA Only App workflow. First Hybridization, First Capture and Second Hybridization and Second Capture section can be run together with the Amplify Enriched Library section.

In this application note, demonstration of the Illumina TruSight Oncology 500 DNA Only App on Biomek NGeniuS system is shown for DNA input concentrations of 40, 80 and 120 ng, with samples ranging in quality from intact genomic DNA to highly compromised FFPE DNA.

Materials and Methods

1.1 Run Setup

DNA samples **(Table 1)** were diluted to the recommended concentration for either 40 ng, 80 ng, or 120 ng inputs according to the instructions in the TruSight Oncology 500 Reference Guide.

Sample	Vendor	Part Number	Sample Integrity (DIN)
Seraseq® Tri-Level Tumor Mutation DNA Mix v2 HC	SeraCare	0710-0097	N/A
Quantitative Multiplex Reference Standard fcDNA (mild)	Horizon Discovery	HD798	≥ 5.1
Quantitative Multiplex Reference Standard fcDNA (moderate)	Horizon Discovery	HD799	2.5-5.0
Quantitative Multiplex Reference Standard fcDNA (severe)	Horizon Discovery	HD803	≤ 2.0

Table 1. Sample types used in preparations of samples for Illumina TruSight Oncology 500 DNA Only.

DNA samples were sheared using a Covaris S220 instrument using microTUBE AFA Fiber Pre-Slit Snap-Cap (Covaris Part Number 520045) in conjunction with the microTUBE Holder (Covaris Part Number 500114) with the following settings:

Setting	Value
Peak Incident Power	175 watts
Duty Factor	10%
Cycles Per Burst	200
Treatment Time	280 seconds
Temperature	7°C
Intensifier	Yes
Other	Intensifier
Pulse Repeats	Not applicable
Average Power	Not applicable

Table 2. Covaris shearing settings used for shearing DNA samples for Illumina TruSight Oncology 500 DNA Only.

When samples are ready, the run is set up in the Biomek NGeniuS customer portal. The Illumina TruSight Oncology 500 DNA Only App has no settings for the user to interact with other than selecting the number of samples to include in the batch (any number from 4 to 16). In order to facilitate operation of the Illumina TruSight Oncology 500 DNA Only App, an App Template Setup Guide (Biomek NGeniuS Illumina TruSight Oncology 500 DNA Only Automation Kit App Template Setup Guide) was created. This document provides details on instrument operation, setup, reagent thawing, and other details to aid operators in working with the Illumina TruSight Oncology 500 DNA Only App. A summary of experiment details is presented in **Table 3**.

Experiment	1	2	3
Sample count	4	9	16
Library Prep Input Mass (ng)	120	80	40
Number of SeraCare samples	3	5	9
Number of Horizon HD798 samples	0	1	2
Number of Horizon HD799 samples	0	1	2
Number of Horizon HD803 samples	0	1	2

Table 3. Summary of experiment conditions and App Settings. All experiments include 1 negative control.

1.2 Reagents, Consumables, and Equipment

Reagents	Manufacturer	Part Number
TruSight Oncology 500 DNA Automation Kit (16 indexes, 64 Samples)	Illumina	20045504
NextSeq 500/550 High Output Kit v2.5 (300 Cycles)	Illumina	20024908
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33231
PCR grade Water	Invitrogen-Life Technology	10977-015
Ethanol	Thermo Fisher Scientific	BP2818-500

Table 4. Reagents used in preparation of libraries with Illumina TruSight Oncology 500 DNA Only and sequencing on Illumina sequencer.

Equipment	Manufacturer
Biomek NGeniuS Sample Prep System	Beckman Coulter Life Sciences
NextSeq 500 Sequencer	Illumina
Qubit	Thermo Fisher Scientific

Table 5. Equipment used in sample preparation and processing of Illumina TruSight Oncology 500 DNA Only.

Consumable	Manufacturer/ Part Number
Foil Plate Seals	Beckman Coulter 538619
Biomek NGeniuS Reaction Vessel, 24 Well	Beckman Coulter C62705
Biomek NGeniuS Lid, 24 Well	Beckman Coulter C62706
Biomek NGeniuS Bulk Reservoirs, 25 mL/Section	Beckman Coulter C62707
Biomek NGeniuS Seal Pad	Beckman Coulter C70665
1025 µL Conductive Filtered Tips, Case	Beckman Coulter C59585
70 µL Conductive Filtered Tips, Case	Beckman Coulter C62712
Empty Tip box 1025 µL, Case	Beckman Coulter C70672
Empty Tip box 70 µL, Case	Beckman Coulter C70673

Table 6. NGeniuS consumables required for sample processing.

1.3 NGeniuS-Produced Libraries and Sequencing

SeraCare and Horizon Discovery samples **(Table 1)** were processed on the Biomek NGeniuS system using reagents, equipment, and consumables detailed in **Tables 4, 5, and 6**. Prior to performing the Library Normalization section, the libraries were quantified using the Qubit 1X dsDNA HS assay kit (Thermo Fisher Scientific) to determine library concentration. Following Library Normalization, the libraries were sequenced on the Illumina NextSeq 500 over the course of two sequencing runs using NextSeq 500/550 High Output Kit v2.5 (300 Cycles) sequencing kits. The 40 ng (16 samples) batch was sequenced on one sequencing run, while the 80 ng (9 samples) and 120 ng (4 samples) batches were combined and sequenced on the second sequencing run. Following sequencing, data was analyzed on Illumina BaseSpace using the DRAGEN TSO500 Solid Eval App (version 2.1.0) and visualized using JMP (version 14.2).

Results & Discussion

The 40 ng input batch run produced 15 libraries (plus one negative control) with a mean pre-library normalization concentration of 33 ng/µL. The 80 ng input batch run produced 8 libraries (plus one negative control) with a mean pre-library normalization concentration of 32.5 ng/µL. The 120 ng input batch run produced 3 libraries (plus one negative control) with a mean pre-library normalization concentration of 28.5 ng/µL. Negative controls in all runs had a mean pre-library normalization concentration of 0.5 ng/µL, well below the required limit of a pre-library normalization concentration of 3 ng/µL. Pre-normalization library yields are shown in **Figure 2**.

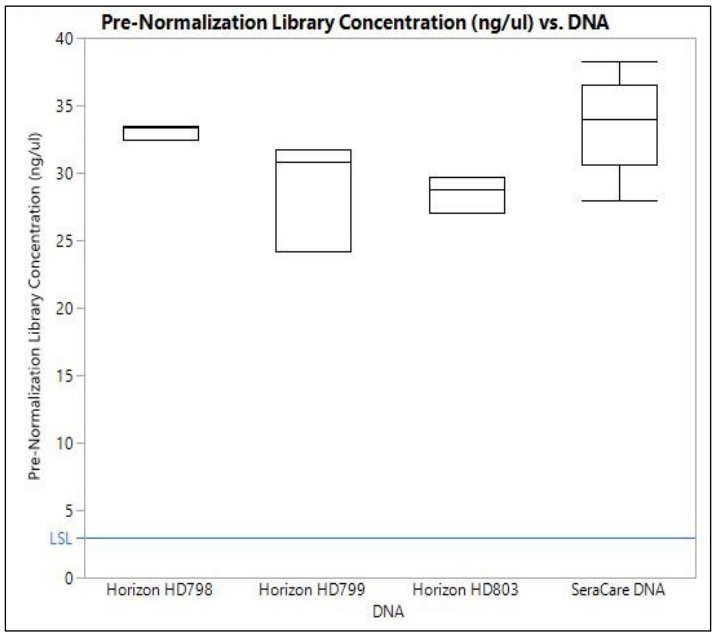

Figure 2. Pre-normalization library concentrations by DNA sample type across all runs.

The first sequencing run containing the 16 libraries of the 40 ng input batch run generated 205,866,836 pass filter reads (92.66% of total reads), of which 85% of reads were successfully identified. 94.35% of all bases were Q30 or higher. Total sequencing output was 88.25 Gb. The second sequencing run containing the nine libraries of the 80 ng input batch run and the four libraries of the 120 ng input batch run generated 271,579,046 pass filter reads (86.54% of total reads), of which 87.29% of reads were successfully identified. 91.37% of all bases were Q30 or higher. Total sequencing output was 115.45 Gb.

Analysis of the sequencing data using the DRAGEN TSO500 Solid Eval App on Illumina BaseSpace showed robust and consistent results across all DNA sample types and input concentrations, while negative controls across all three runs failed to generate any useful sequencing metrics. Mean percent read alignment for all DNA sample types was greater than 96%, while mean percent read enrichment for all DNA sample types was 89%. Percent read alignment and percent read enrichment by DNA sample type for all runs are shown in **Figure 3**.

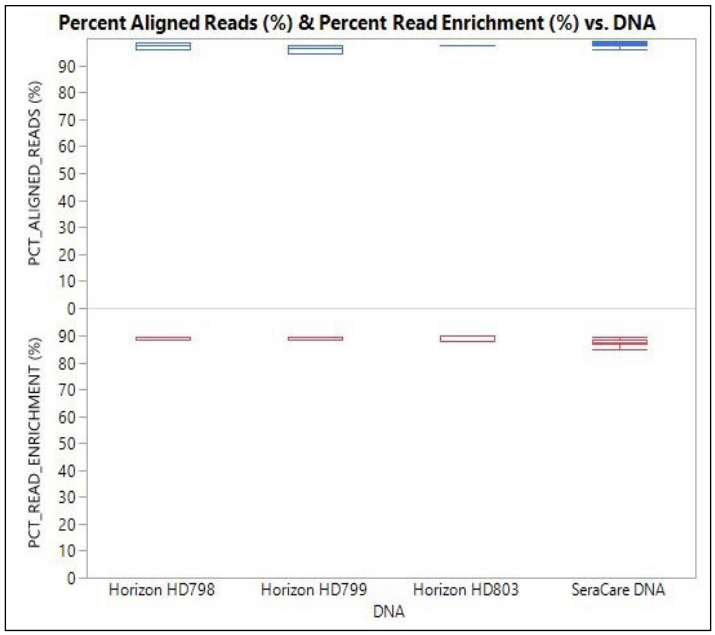

Figure 3. Percent aligned reads and percent read enrichment by DNA sample type across all runs.

Median insert size of the library fragments was consistently higher than the lower specification limit of 70 bp for this limit. There is a predictable decline in median insert size of the library fragments in the Horizon Discovery samples, as the intensity of the formalin treatment increases from the HD798 (mild) through to HD803 (severe), reflecting the increased fragmentation of the input material. Median insert by DNA sample type for all runs are shown in **Figure 4**.

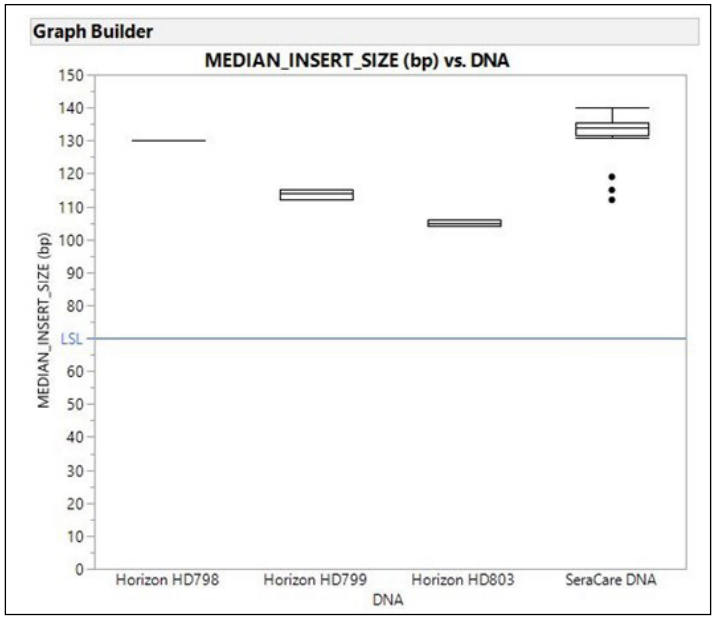

Figure 4. Median insert size by DNA sample type across all runs. The three outliers in the SeraCare DNA column represent the three 120 ng SeraCare replicates and the slightly less efficient shearing parameters used for that input mass.

The DRAGEN TSO500 Solid Eval App evaluates the presence of contaminating DNA in each library by comparing allele frequencies of SNVs detected in the sequencing data with a known collection of SNVs. As the Horizon Discovery samples represent a collection of different cell lines in each sample, an inflated contamination score that exceeds the upper specification limit of 1457 is observed in these samples, as opposed to the SeraCare DNA samples which represent a single discrete cell line. Contamination scores by DNA sample type for all runs are shown in **Figure 5**.

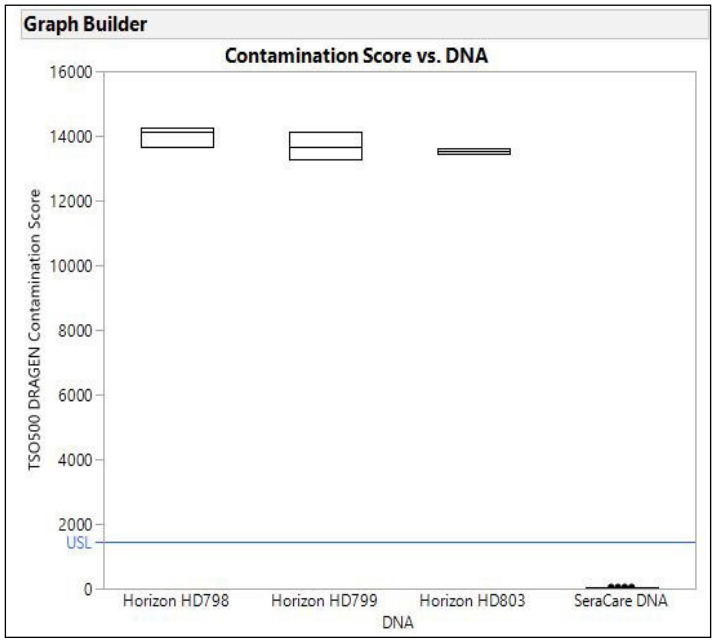

Figure 5. Contamination Score by DNA sample type across all batch runs. Horizon Discovery samples represent a variety of different cell lines in a single sample, resulting in a high variety of background allele frequencies that are flagged as contamination by the DRAGEN TSO500 Solid Eval App. The SeraCare samples represent a single cell line, and as such are well below the Contamination Score upper specification limit.

Excellent coverage depth was achieved across the runs, with the lowest median exon coverage of any library at 375X. Uniformity of coverage was consistent across all DNA sample types, with the percent exon coverage at 50X greater than 99% for all libraries (lower specification limit for this metric is 90%). Percent Exons at 50X by DNA sample type for all runs is presented in **Figure 6**.

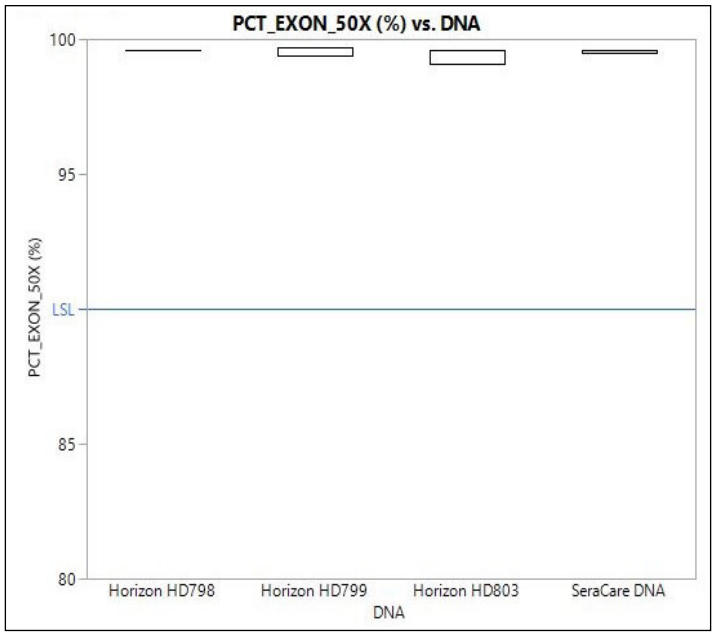

Figure 6. Percent Exons at 50X coverage by DNA sample type across all batch runs.

Usable MSI sites represent the number of sites in the sequencing data that can be used to calculate the sample's MSI status. For this metric, a lower specification limit of 40 sites is used. The lowest number of usable MSI sites in any library across all three runs was 84, with a mean number of usable MSI sites of 116 across all libraries. Usable MSI sites by DNA sample type for all runs is presented in **Figure 7**.

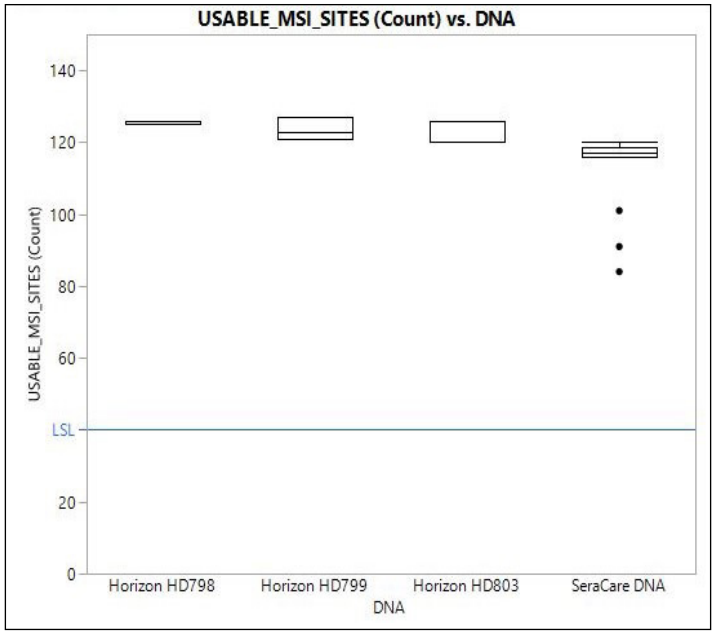

Figure 7. Usable MSI sites by DNA sample type across all batch runs. The three outliers in the SeraCare DNA column represent the three 120 ng SeraCare replicates and the slightly less efficient shearing parameters used for that input mass.

Tumor mutation burden (TMB) represents the number of nonsynonymous mutations within the coding region of a tumor genome after filtering variants based on a number of different parameters, including a minimum variant allele threshold. The presence of multiple cell lines in the Horizon Discovery samples contributes to a much higher background signal of variants, and as such the Horizon Discovery samples have much higher calculated TMB scores compared to the SeraCare DNA samples, which represent a single discrete cell line. TMB scores by DNA sample type for all runs is presented in **Figure 8**.

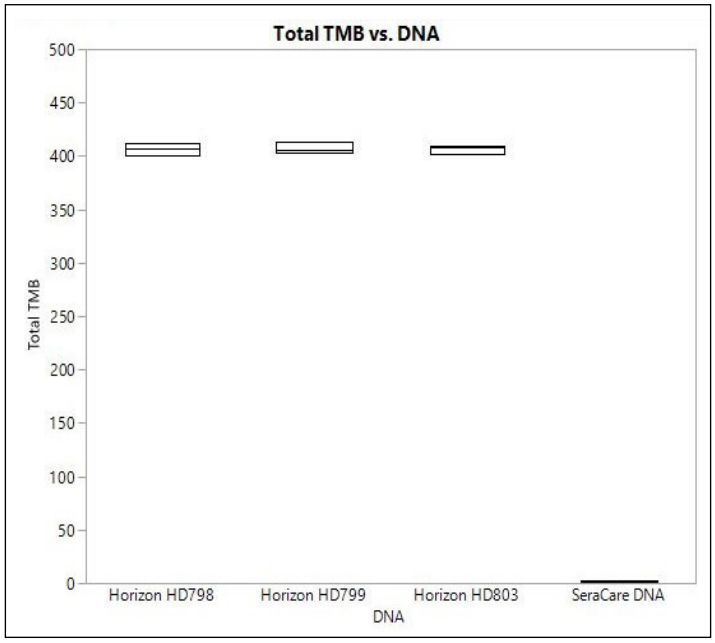

Figure 8. TMB scores by DNA sample type across all batch runs.

TruSight Oncology 500 reports small DNA variants with 95% analytical sensitivity down to 5% variant allele frequency (VAF) at recommended inputs of 40 ng or higher. The SeraCare and Horizon Discovery DNA samples contain variants with allele frequencies ranging from 4% to 10% in the case of the SeraCare DNA samples and from 1% to 24.5% in the Horizon Discovery DNA samples. For the purposes of this analysis small DNA variants were grouped into three groups consisting of variants with an allele frequency of 10% or higher, variants with allele frequencies ranging from 5% to 9%, and variants with allele frequencies 4% or lower. Over the course of all runs, libraries prepared using the Illumina TruSight Oncology 500 DNA Only App on the Biomek NGeniuS system were able to detect 98% of all small DNA variants with a 5% variant allele frequency or higher. Small DNA variant detection by DNA sample type for all runs is presented in **Figure 9**.

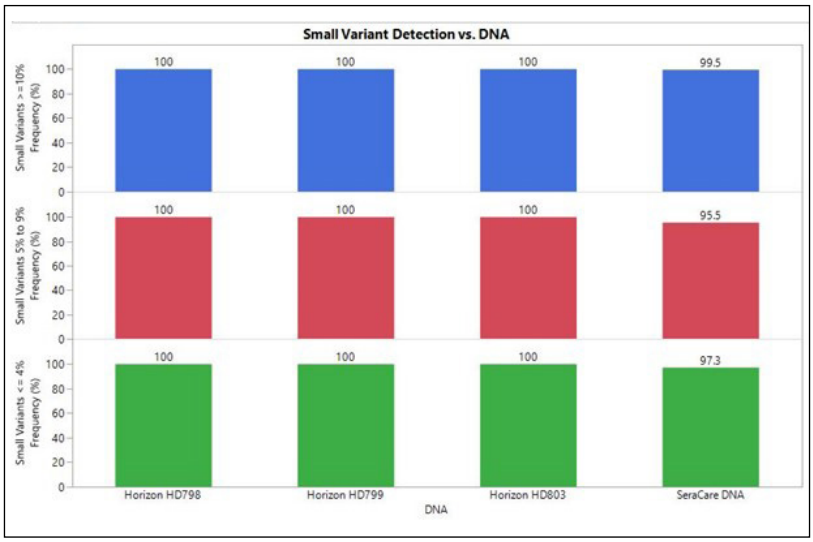

Figure 9. Small variant detection by DNA sample type across all batch runs.

Summary

We demonstrated that libraries produced using the Illumina TruSight Oncology 500 DNA Only App on the Biomek NGeniuS Next Generation Library Prep System produce high-quality libraries that meet the rigorous specifications of the Illumina TruSight Oncology 500 assay across a range of input amounts and sample quality.

References

1. Illumina TruSight Oncology 500 Reference Guide (Document # 1000000067621 v10)
2. Biomek NGeniuS Illumina TruSight Oncology 500 DNA Only Automation Kit App Template Setup Guide (2023-GBL-EN-101806-v1)
3. DRAGEN TruSight Oncology 500 Analysis Software v2.1 Local (Document # 200031722 v01)
4. Input Recommendations for TMB, MSI, and Small Variant Analysis with TruSight Oncology 500 (1170-2019-001-B)

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