Application Note

Agilent
Trusted Answers

Comparisons among Six Different Library Preparation Kits using FFPE Samples for Exome Enrichment Applications

Authors

Jesse D. Luce,
Prashant K. Singh,
Sean T. Glenn
Department of Cancer Genetics and Genomics,
Roswell Park Cancer Institute,
Buffalo, NY

Josh Zhiyong Wang,
Bahram Arezi,
Bilan Hsue
Agilent Technologies,
Santa Clara, CA

Abstract

Exome sequencing and targeted panel sequencing are popular next generation sequencing (NGS) approaches to identify and characterize different genetic aberrations in research and clinical samples, such as cancer specimens. Many factors including panel targets design, wet lab handling and bioinformatic analysis can affect the sensitivity and accuracy of variant/aberration detection. Here we report the comparison results among six different library preparation kits from multiple vendors using the same FFPE samples. At 100X raw sequencing depth, the Agilent SureSelect$^{XT\ HS}$ Library Preparation kit generated the highest performance on four key metrics: on-target rate, average read depth, percentage of target bases at 20X read depth and the yield of pre-capture library. Vendor A kit ranked second in two metrics of percentage of target bases at 20X read depth and pre-capture library yield; Vendor E ranked second in two metrics of on-target rate and average read depth. Overall, the Agilent SureSelect$^{XT\ HS}$ kit outperformed the other 5 kits evaluated.

Introduction

Detection of genetic variations presented in DNA samples is a vital step in both basic and clinical research. Targeted sequencing and exome sequencing are great tools to investigate samples with complex mutation profiles such as tumor samples. Focusing on selected genes or gene regions associated with disease or phenotype enables researchers to sequence at a much deeper depth in a cost-effective way.

Among different target enrichment workflows, hybridization-based target enrichment provides great flexibility and high-quality sequencing data. A standard target enrichment workflow includes pre-capture DNA library preparation, hybridization capture of regions of interest, and post-capture PCR to generate sufficient library for sequencing. To maximize the efficiency of hybridization capture, the pre-capture library must have sufficient yield with high complexity. Different commercial library preparation kits utilize different enzymes, reagents and workflows to optimize pre-capture library preparation efficiency.

The Agilent SureSelect$^{XT\ HS}$ library preparation kit[1] is optimized for intact and FFPE samples. It has a 8-hour turnaround time and only need as low as 10 ng of DNA input. The molecular barcode feature enables accurate and sensitive detection of variants with low allele frequency. A recent study showed that the SureSelect$^{XT\ HS}$ workflow was able to achieve highly sensitive (detection limit ~0.1%) and easily customizable ctDNA sequencing[2].

In this application note, we compared library preparation kits from six different vendors. Pre-capture libraries were made from 4 degraded FFPE samples and then hybridized to Agilent SureSelect Human All Exon V6. Captured libraries were sequenced on the Illumina HiSeq2500. Several sequencing QC metrics were used to determine the performance of these kits.

Materials and Methods

Samples and Library Preparations

Four melanoma FFPE samples designated as FFPE1, FFPE2, FFPE3 and FFPE4 were used in this study. The quality (ddCq value) of the samples was determined using the Agilent NGS FFPE QC kit, (ddCq ranging from 0.23 to 2.32). DNA was sheared with a Covaris S220 Focused-ultrasonicator. The Agilent 4200 TapeStation was used in all QC steps to assess DNA fragment sizes throughout shearing, library preparation and post-capture PCR steps.

Libraries were prepared using SureSelect$^{XT\ HS}$ and five other library preparation kits identified as Vendors A, B, C, D and E for a total of six sets of libraries. After pre-capture libraries were generated, hybridization capture was performed with the Agilent SureSelect Human All Exon v6 following the manufacturer's protocol. The post-capture PCR cycle number was set at 9. Agilent product information is listed in Table 1.

Table 1. Agilent products used in this study

Product name	Catalog number
SureSelectXT HS Reagent Kit, index 1-16 + Human All Exon v6 Target Enrichment Baits, 16 rxn	G9704K
SureSelectXT HS Reagent Kit, index 17-32 + Human All Exon v6 Target Enrichment Baits, 16 rxn	G9705K
SureSelectXT HS Reagent Kit, index 1-32 + Human All Exon v6 Target Enrichment Baits, 96 rxn	G9706K

The final libraries were pooled and loaded at 16 pM for on-board cluster generation of multiple Rapid Mode v2 flow cells then sequenced on an Illumina HiSeq 2500 System for 2X 100 cycles at a depth of ~40-80 M reads per sample. Illumina CASAVA 1.8.2 software was used to perform base calling and demultiplexing.

Data Analysis

Filtered reads were normalized to 60 million reads to achieve 100X sequencing depth and subsequently mapped to the reference human genome (hg19) with BWA aligner. Three libraries failed to generate 60 million reads, including FFPE2 with Vendor A and Vendor C and FFPE3 with Vendor C. Sequencing metrics including on-target percentage and read depth were analyzed with bedtools (http://bedtools.readthedocs.io/en/latest/). Agilent SureCall software was used to analyze molecular barcodes for Agilent SureSelect$^{XT\ HS}$ libraries.

Results and Discussion

Comparison of Pre-capture Library Yields for Six Different Kits

As FFPE DNA samples can vary greatly in their quality or the extent of degradation, DNA quantification of an FFPE DNA sample by Qubit doesn't reflect amplifiable quantity, a metric that is critical in many NGS applications involving PCR. Thus, a qPCR-based method to determine the quality and amplifiable quantity of FFPE DNA samples is a commonly used method to generate a normalized DNA integrity score (ddCq), with a lower ddCq value indicating a better quality sample that can be readily amplified. The four FFPE DNA samples we selected for this comparison study were designated FFPE1, FFPE2, FFPE3 and FFPE4, with respective ddCq values of 0.23, 0.57, 2.08 and 2.32. For this comparison study, 100 ng of input DNA from each FFPE sample was used, based on their Qubit concentrations. No adjustment of DNA input amounts was made, based on their ddCq values, since all of the non-Agilent kit protocols did not call for adjustment of input DNA amounts, based on ddCq values.

The SureSelect$^{XT\ HS}$ kit was included in this study, together with 5 other non-Agilent library preparation kits designated as Vendors A, B, C, D and E. Note that the experimental protocols for these six kits contain seven similar steps: genomic DNA shearing, end-repair, dA tailing, adaptor ligation, pre-capture PCR, capture hybridization and post-capture PCR for the final library. However, the protocols differ considerably in the number of Agencourt® AMPure® XP purification steps performed (between 2 and 6), incubation time for each enzymatic step, PCR cycle numbers, and therefore the total hands-on time for library preparation.

Table 2 compares the pre-capture library yields of these 4 FFPE samples using six library preparation kits. The number of PCR cycles affects the overall library yield, with higher cycle number resulting in higher yields. Three kits (Vendor A, Vendor D and Vendor E) used 10 cycles per their protocol recommendations,

while three other kits (Agilent SureSelect$^{XT\ HS}$, Vendor B, Vendor C) used 12 cycles per their protocols. In general, all kits produced better PCR yields with samples FFPE1 and FFPE2 than they produced with samples FFPE3 or FFPE4. This was presumably due to FFPE1 and FFPE2 having lower ddCq values and thus higher DNA integrity, making them more suitable for PCR. Using 10 PCR cycles, the kit from Vendor A generated higher library yields than the kits from Vendor D and Vendor E. Using 12 PCR cycles, the Agilent SureSelect$^{XT\ HS}$ kit generated much higher pre-capture library yields than the kits from Vendor B and Vendor C. In fact, the Agilent SureSelect$^{XT\ HS}$ kit yielded at least 2.5 times and as much as 6 times as much pre-capture library DNA as any of the five competitors' kits.

Table 2. Pre-capture library prep yield comparison (nanograms of DNA)

	ddCq	Vendor A	Vendor B	Vendor C	Vendor D	Vendor E	Agilent XT HS
PCR cycle		10	12	12	10	10	12
FFPE1	0.23	1052	398	476	316	588	**2580**
FFPE2	0.57	633	358	540	251	386	**1605**
FFPE3	2.08	325	161	464	189	296	**762**
FFPE4	2.32	355	139	378	278	355	**2100**

Overall, the Agilent SureSelect$^{XT\ HS}$ and Vendor A kits were the top two performers among all six in terms of pre-capture library prep yield. Using the default parameters in the protocol, the Agilent SureSelect$^{XT\ HS}$ kit generated robust pre-capture library yields across all 4 FFPE samples. Sample FFPE3 had a somewhat lower yield at 762 ng, but it was sufficient for capture hybridization.

Comparisons of % On-target Sequencing

For the sequencing data from SureSelect$^{XT\ HS}$ libraries, additional analysis was performed using Agilent SureCall software to include molecular barcode information reads. These data are indicated as "Agilent XT HS MBC" in this application note (Figures 1-3).

The percentage of reads in the exome regions is a useful metric when evaluating the performance of an exome target enrichment workflow. When using high-quality samples from the International HapMap Project and the Agilent SureSelect Human All Exon V6 capture library, one can typically achieve a 70-85% on target rate. Figure 1 shows the on-target percentage comparison for all libraries generated by the kits in this study. For SureSelect$^{XT\ HS}$ and SureSelect$^{XT\ HS}$ MBC data, the on-target percentage for all 4 FFPE samples is consistently between 75-85%, even though FFPE3 and FFPE4 are of much lower quality than FFPE1 or FFPE2, based on their ddCq values. These results demonstrate that the SureSelect$^{XT\ HS}$ kit maintained a high level of performance (in terms of % on-target) across all FFPE samples of varying quality, and can significantly outperform other kits on the % on-target metric when using more degraded FFPE samples (Figure 1).

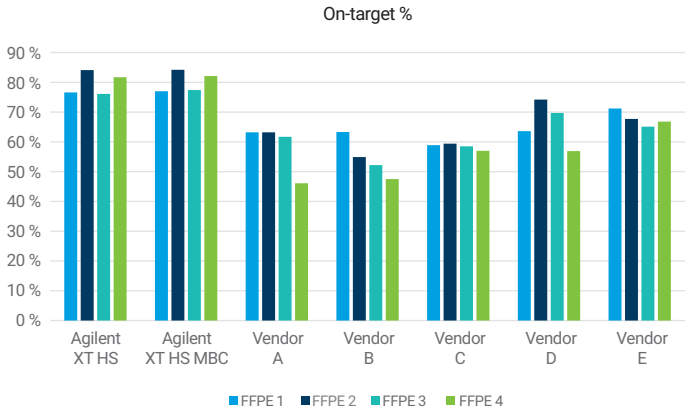

Figure 1. DNA sequencing on-target percentage comparison

In contrast, library preparation kits from other vendors showed lower % on-target results. The kit from Vendor E performed the best among them, producing a consistent % on-target of ~65-71%, which is as much as 10-15% lower than what was achieved using the Agilent SureSelect$^{XT\ HS}$ kit. The on-target percentages using the competitors' kits for the two lower quality (FFPE3 and FFPE 4) samples were lower than the on-target percentages for the higher quality FFPE1 and FFPE 2 samples. For example, on-target rates of 46.1% and 47.5% were obtained for the FFPE4 samples when using the Vendor A and Vendor B kits, respectively. The Vendor C kit generated consistent on-target rates from 57% to 59.4% across the four FFPE samples.

Comparison of Average Read Depth and Percentage of Target Bases at 20X Read Depth

The main purpose of a targeted NGS experiment is to generate sufficient read depth in the genomic regions of interest so that variations of reference sequences can be identified at high confidence. Thus, evaluation of performance based on achieved average sequencing read depth as well as percentage of target bases at a certain read depth (10X, 20X or 100X, etc.) are the most important QC metrics when determining the effectiveness of a target enrichment solution. As the same bait set (SureSelect Human All Exon v6) was used, the observed differences would be attributed to the library prep kits used for the comparison, and not the capture hybridization bait set.

Usually, the desired sequencing read depth for an experiment depends on the allele frequency of expected variants. In constitutional sample NGS experiments where homozygous or heterozygous variants are expected, a read depth of 20X is considered sufficient to result in a high-confidence call of the

variant. However, in cancer sample NGS experiments, where low allele frequency variants are expected, a read depth of 200X or 1000X or even 20,000X together with molecular barcode analysis may be needed to achieve a high-confidence call on the variant[2].

Nevertheless, in a sequencing experiment, a higher percentage of target bases at 20X read depth would typically correlate with a higher percentage of target bases exceeding 100X or 1000X read depth as well. Therefore, in this application note, although FFPE cancer samples were used for exome sequencing, average read depth and percentage of target bases at 20X read depth were used as the QC metrics to compare the six different library prep kits.

Table 3 shows the average read depth and percentage of target bases at 20X read depth data for all kits and all four FFPE samples. Figure 2 shows the average read depth comparison with most samples' raw sequencing read depth at 100X, except for the 3 samples mentioned earlier (Materials and Methods), which are at 75X – 85X sequencing depth. For FFPE1 and FFPE2 samples, the Agilent SureSelect$^{XT\ HS}$ kit exhibited the highest read depth (62X and 71X) among all kits (Table 3), with the Vendor D kit (52X and 65X) and Vendor E kit (60X and 56X) tied for the second highest read depth. For the FFPE3 sample, the Vendor D kit (52X) and Vendor E kit (46X) provided the highest average read depths. However, for the FFPE4 sample, the Agilent SureSelect$^{XT\ HS}$ kit was once again the best performer, generating 60X average read depth, with the Vendor E kit (52X) being the second best.

Figure 3 shows the comparison of the percentage of target bases at 20X read depth for all six kits. For the FFPE1 and FFPE2

Table 3. Comparison of average read depth and percentage of target bases at defined read depths

	Agilent XT HS				Agilent XT HS MBC			
	FFPE1	FFPE2	FFPE3	FFPE4	FFPE1	FFPE2	FFPE3	FFPE4
reads in	60M	60M	60M	60M	60M	60M	60M	60M
Average Read Depth:	62	71	39	60	55	64	39	55
...at least 1 read:	97.7%	97.3%	97.4%	97.4%	97.7%	97.3%	97.3%	97.3%
...at least 10 reads:	94.6%	92.8%	92.8%	91.6%	93.8%	91.8%	92.1%	90.4%
...at least 20 reads:	87.0%	85.1%	81.2%	80.3%	84.3%	82.7%	79.4%	77.5%
...at least 50 reads:	51.7%	56.7%	29.5%	45.7%	45.3%	51.0%	28.8%	41.2%

	Vendor A				Vendor B			
	FFPE1	FFPE2	FFPE3	FFPE4	FFPE1	FFPE2	FFPE3	FFPE4
reads in	60M	46.6M*	60M	60M	60M	60M	60M	60M
Average Read Depth:	51	41	42	34	49	44	28	26
...at least 1 read:	97.8%	97.7%	97.4%	97.4%	97.7%	97.6%	97.2%	97.2%
...at least 10 reads:	95.3%	94.2%	93.8%	87.2%	94.3%	94.3%	88.5%	83.3%
...at least 20 reads:	86.2%	82.1%	82.6%	62.1%	82.4%	83.3%	65.5%	54.7%
...at least 50 reads:	40.3%	28.1%	30.2%	19.7%	37.1%	33.4%	9.9%	10.6%

** 78X sequencing depth*

	Vendor C				Vendor D				Vendor E			
	FFPE1	FFPE2	FFPE3	FFPE4	FFPE1	FFPE2	FFPE3	FFPE4	FFPE1	FFPE2	FFPE3	FFPE4
reads in	60M	51M*	45M**	60M	60M	60M	60M	60M	60M	60M	60M	60M
Average Read Depth:	51	44	33	42	52	65	52	43	60	56	46	52
...at least 1 read:	97.8%	97.7%	97.3%	97.5%	97.2%	97.3%	96.7%	95.8%	97.1%	97.0%	96.5%	96.3%
...at least 10 reads:	95.2%	94.5%	89.9%	91.1%	82.8%	88.0%	83.6%	72.2%	82.3%	83.9%	81.6%	77.3%
...at least 20 reads:	84.9%	82.3%	70.5%	74.0%	62.7%	72.2%	65.4%	50.6%	63.5%	65.1%	62.3%	56.4%
...at least 50 reads:	39.2%	31.1%	17.7%	28.7%	34.2%	41.4%	34.2%	27.1%	37.3%	35.5%	30.7%	31.1%

** 85X depth ** 75X sequencing depth*

samples, the Agilent SureSelect$^{XT\ HS}$ kit showed the best performance at 87% and 85.1%, respectively, with the Vendor A kit (86.2% and 82.1%) a close second, and the Vendor C kit (84.9% and 82.3%) as well as the Vendor B kit (82.4% and 83.2%) close behind. The performance for either the Vendor D or Vendor E kit was substantially lower (62% - 72%), which is surprising, because Vendor D and Vendor E kits performed well in average read depth. A likely explanation is that the Vendor D and Vendor E kits don't generate good uniformity across targeted bases, and the better average read depths were achieved by a subset of target bases having very high depth coverage.

For the FFPE3 and FFPE4 samples, which are the two samples with the worst ddCq values, the Agilent SureSelect$^{XT\ HS}$ kit also provided the best performance at 81.2% and 80.3% coverage of target bases at 20X read depth, respectively. The Vendor A kit (82.6% and 62.1%) and the Vendor C kit (70.5% and 74.0%) delivered the second and third highest percentage of target bases covered. Both Vendor B and Vendor C kits had similar performance with the FFPE1 and FFPE2 samples, but very different performance when using FFPE3 and FFPE4 samples. This suggests that the Vendor C kit is better at handling more degraded FFPE samples than the Vendor B kit (Figure 3). Overall, the Agilent SureSelect$^{XT\ HS}$ kit was the only kit that consistently enabled at least 80% coverage of target bases at 20X read depth, with 100X sequencing depth across all 4 FFPE samples of varying quality. Additionally, the Agilent SureSelect$^{XT\ HS}$ kit provided the highest percentage of target bases at 50X read depth, demonstrating superior performance uniformity.

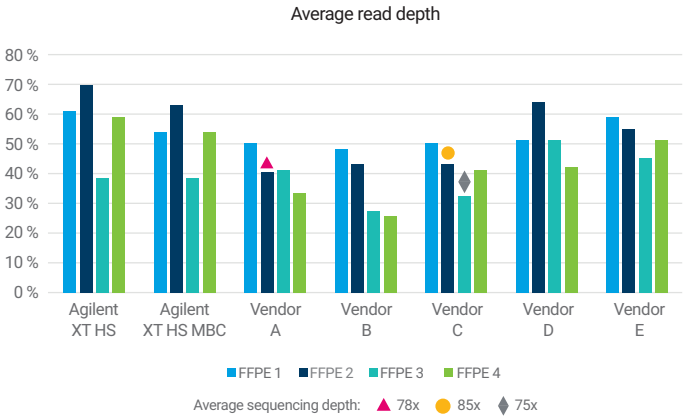

Figure 2. Average read depth comparison at 100X sequencing depth (60 million reads), except where indicated

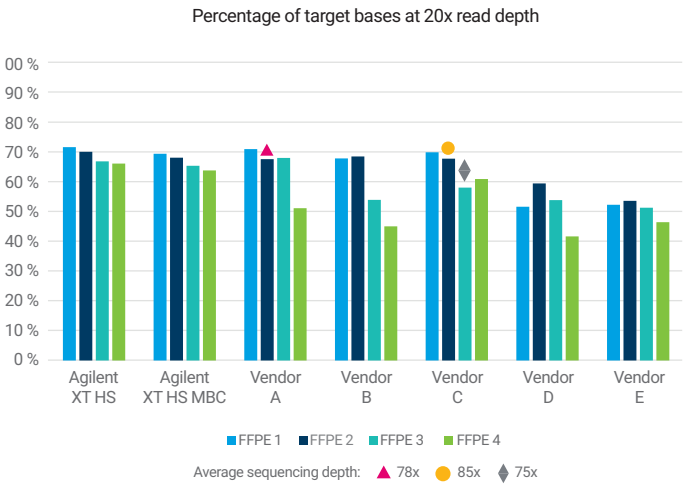

Figure 3. Percentage of target bases at 20X read depth and 100X sequencing depth (60 million reads), except where indicated otherwise

Additional Features of the Agilent SureSelect$^{XT\ HS}$ Kit

This study used 100 ng of input DNA for library preparation, based on Qubit values, regardless of the sample's degradation state, in order to evaluate all the kits under the same parameters. It should be noted that Qubit values do not reflect the amplifiable amount of DNA present, and the determination of input DNA amount should also be based on other DNA Integrity metrics such as ddCq values or DNA Integrity Number (DIN). Of the six kits that were evaluated, the Agilent SureSelect$^{XT\ HS}$ kit is the only one that provided guidelines for adjusting input amounts and sequencing depth based on DNA quality.

The Agilent SureSelect$^{XT\ HS}$ kit is also the only kit incorporating molecular barcode technology to improve variant calling accuracy and enable highly sensitive detection of low allele frequency variants[2]. Note that the effect of molecular barcode analysis on overall read depth (after deduplication) and error correction (by consensus making) is much more pronounced when higher sequencing read depth (>10,000X) and/or low gDNA input amounts (< = 25 ng) are used[2].

For the Agilent SureSelect$^{XT\ HS}$ kit used in this study, a separate analysis taking advantage of molecular barcodes was also performed. With molecular barcode analysis, the on-target percentage improved slightly (Figure 1). Both average read depth and percentage of target bases at 20X read depth dropped slightly (Figures 2 and 3). This is most likely due to the lower raw sequencing depth (100X) and higher gDNA input amount (100 ng) used in this experiment compared to what was used in reference 2.

References

1. "SureSelect$^{XT\ HS}$ Target Enrichment", Agilent publication number 5991-8165EN.
2. "Ultra-sensitive Cancer Liquid Biopsy Analysis with the Agilent SureSelect$^{XT\ HS}$ Target Enrichment Workflow." Agilent publication number 5991-8464EN.

www.agilent.com

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

PR7000-1892
© Agilent Technologies, Inc. 2018
Printed in the USA, Month April 13, 2018
5991-9293EN

Agilent
Trusted Answers