Application Note

Genomics

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Enzymatic Shearing Method for Agilent Avida Methyl and Duo Workflows

Preserving methylation markers for accurate analysis

Authors

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Abstract

For genomic and epigenomic analyses, the Agilent Avida workflows are optimized to maximize DNA recovery and offer high sensitivity through an innovative, high-performance target enrichment technology. Here, we describe the incorporation of an enzymatic fragmentation method that preserves methylation markers for the Avida Methyl and Duo workflows, using genomic DNA (gDNA) from a cell line, formalin-compromised (fc) samples, and formalin-fixed paraffin-embedded (FFPE) samples. Our results demonstrate that the methylation markers derived from libraries based on enzymatic shearing are retained at the same level compared to those from mechanical shearing in both Avida Methyl and Duo workflows. Furthermore, sequencing metrics such as on-target rate, base coverage, and uniformity are consistent across the two shearing methods. We verified the detection of variants with allele frequencies as low as 1% using a multiplex reference fc standard in the Avida Duo workflow. Finally, evaluation of a tumor FFPE DNA sample showed comparable genomic alterations and methylation changes between the two fragmentation methods.

Introduction

The innovative Agilent Avida target enrichment technology enhances multiomic analyses by enabling highly sensitive detection of low-frequency allele variants and methylation changes. The Avida reagent kits have been optimized for low-input samples, such as cell-free and formalin-fixed paraffin-embedded (FFPE) DNA, making them suitable for applications that require high sensitivity.[1,2]

Using the Agilent Avida Duo workflow, both DNA variants and methylation changes can be captured together from a single sample input, eliminating the need for sample splitting. Alternatively, target regions can be captured separately, using the Agilent Avida DNA workflow for DNA target regions, or the Agilent Avida Methyl workflow for methyl target regions (Figure 1).

The standard protocols for the Avida workflows recommend mechanical shearing for DNA fragmentation prior to library preparation. While effective, mechanical shearing is costly, requires extra instrumentation, poses challenges for automation integration, and may lead to sample loss and potential DNA damage. To address these challenges, we evaluated an enzymatic shearing method and demonstrated that the Avida DNA workflow was compatible with the Agilent SureSelect Enzymatic Fragmentation kit.[3] However, this kit was not compatible with the Avida Methyl and Duo workflows, presumably due to the loss of methylation markers resulting from nick-translation activity.

In this study, we examine the effectiveness of an alternative enzymatic fragmentation method in preserving methylation markers for use in the Avida Methyl and Duo workflows. A range of samples were analyzed (intact gDNA, fcDNA, FFPE DNA). For the Avida Duo workflow, we used two DNA panels for DNA target enrichment: the Agilent Avida DNA Expanded Cancer panel, covering over 100 genes, and the Agilent Avida DNA Discovery Cancer panel, covering 682 genes. For the Avida Methyl and Avida Duo workflows, the Avida Methyl 3400 DMR Cancer panel, covering approximately 3400 differentially methylated regions (DMRs), was used.

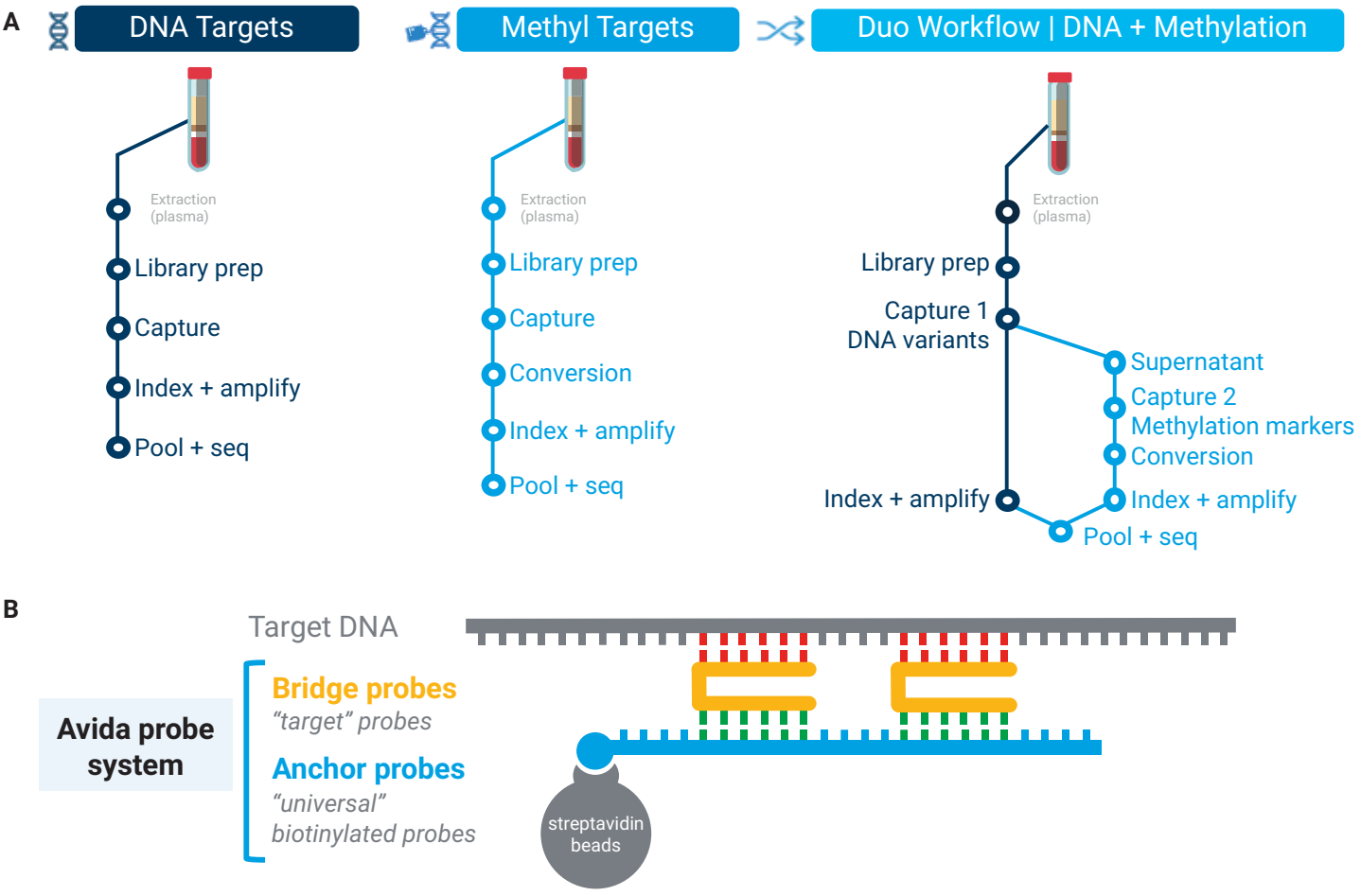

Figure 1. (A) Schematic representation of the Agilent Avida DNA, Methyl, and Duo workflows for preparing sequencing-ready libraries. Targets can be captured individually as DNA or Methyl targets in separate workflows (left, middle), or together as both DNA and Methyl targets in one workflow (right). (B) The proprietary Avida capture technology enables a faster, more efficient hybridization reaction using a dual probe system. The captured target is stabilized when multiple short bridge probes synergistically hybridize to both the target DNA and the biotinylated anchor probe, ensuring robust and precise target enrichment.

Experimental

Sample types

Samples used in this study included Haplotype Map gDNA (intact gDNA, NA24385, DNA integrity number [DIN] 9.2, Coriell Life Sciences), formalin-compromised (fc) multiplex reference standard DNA (HD799, DIN 4.2, Horizon Discovery), and DNA derived from an FFPE lung sample (DIN 6.7, BioChain Institute Inc.). The quality of the gDNA was assessed using the Agilent 4200 TapeStation system and Agilent Genomic DNA ScreenTape assay. For FFPE DNA sample preparation, it is recommended to use a suitable purification system, such as Qiagen's QIAamp DNA FFPE Tissue Kit and Qiagen's Deparaffinization Solution, following the manufacturer's protocols.

Fragmentation

For mechanical shearing, the process was conducted in 50 µL low-Tris-EDTA (TE) buffer using a Covaris E220 device, following the Agilent Avida Methyl reagent kit protocol (p/n G9419-90000) or the Agilent Avida Duo reagent kit protocol (p/n G9439-90000) (Figure 2). Enzymatic shearing was carried out using NEBNext UltraShear (NEB 7634S/L, NEB BioLabs Inc) in a reaction volume of 44 µL (Figure 2). Briefly, the gDNA was diluted in 26 µL nuclease-free water, and then mixed with 4 µL of enzyme and 14 µL of reaction buffer. The reaction was incubated at 45 °C for 20 minutes, followed by heat inactivation of the enzyme at 65 °C for 15 minutes. Fragmentation by UltraShear upstream of Avida Methyl and Duo library preparation is not recommended for FFPE samples of low quality (DIN <3).

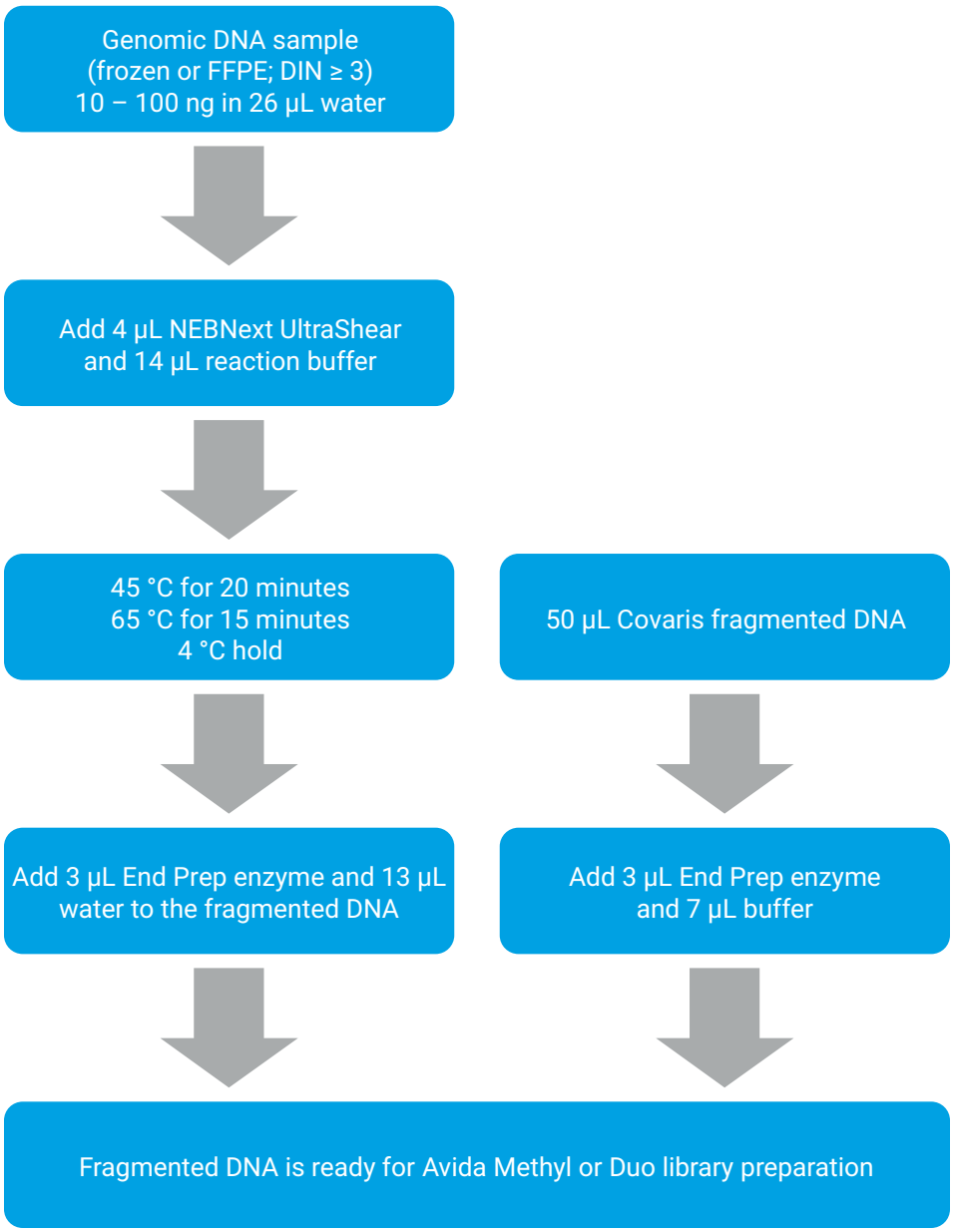

Figure 2. Overview of the shearing protocols tested for the Agilent Methyl and Duo workflows. Enzymatic shearing is shown on the left and mechanical shearing is shown on the right.

Library preparation and target enrichment

Library preparation following enzymatic fragmentation involved small adjustments to the protocol. End-repair and dA tailing was performed by adding 3 µL of End-Prep enzyme and 13 µL nuclease-free water (instead of the 7 µL End-Prep buffer normally used in the Avida Methyl and Duo protocols) to the fragmented gDNA. The end-repaired and dA-tailed DNA fragments were then ligated to the adaptor and subsequently enriched.

In the Avida Methyl workflow, the ligated DNA was enriched using the Avida Methyl 3400 DMR Cancer panel (p/n 5280-0059), which includes approximately 3400 DMRs identified from both public databases and internal sequencing studies, for robust and accurate methylation detection.

In the Avida Duo workflow, the ligated DNA was enriched using either the Avida DNA Expanded Cancer panel (p/n 5280-0047), which encompasses over 100 genes for mutation, copy number, and translocation detection, or the Avida DNA Discovery Cancer panel (p/n 5280-0044), which covers the exonic regions of 682 key genes and translocation hotspot introns, for genomic profiling and biomarker assessment. The hybridization supernatant was then subjected to a second enrichment using the Avida Methyl 3400 DMR Cancer panel followed by Avida soft conversion (Figure 1A).

The captured DNA was washed and amplified to incorporate sample indexes. The quality and quantity of the amplified libraries were assessed using the Agilent 4200 TapeStation system and Agilent D1000 ScreenTape assay. The final libraries were then pooled and sequenced at 2 x 150 read length on the Illumina NovaSeq6000. Sequencing depths were adjusted based on panel size to ensure that the maximum number of unique molecules were sequenced, as illustrated in the figure legends.

Data analysis

FASTQ files were aligned using Bismark for methylation data and Burrows-Wheeler Aligner (BWA) for DNA data, followed by deduplication that accounted for unique molecular identifiers (UMIs). Single-stranded UMI consensus reads were then generated to assess sequencing metrics such as molecule recovery, insert size, uniformity, GC coverage, variant detection, and percentage of methylated CpG. The on-target rate was calculated without considering UMI data. For more details on the analysis pipeline, see the Avida DNA Targeted Sequencing Analysis Technical Guide (p/n G9409-90001) and the Avida Targeted Methylation Sequencing Analysis Technical Guide (p/n G9419-90001).[S1,S2]

Results and discussion

Library yields and median insert size

The enzymatic fragmentation method described here consistently produces higher library yields, ranging from 1.2 to 2.3 times higher, compared to Covaris shearing across various sample types (intact, fc, and FFPE lung DNA), input amounts (10 to 50 ng), and panel sizes and types (Avida DNA Expanded, DNA Discovery, and Methyl 3400 DMR Cancer panels) (Figure 3A, 3B). The fragment sizes of the libraries analyzed using the 4200 TapeStation system and D1000 ScreenTape assay were in the same range (Figure 3A, 3C). These results are similar to the results obtained from our previous study using the Agilent SureSelect Enzymatic Fragmentation kit with the Avida DNA workflow.[3] This suggests that lower yields with Covaris fragmentation are likely due to sample loss during transfer steps and/or DNA damage during mechanical fragmentation.[4]

A key objective of this study was to achieve fragment sizes comparable to those produced by mechanical shearing when using enzymatic fragmentation for the Avida Methyl and Avida Duo workflows. The conditions developed in this study (Figure 2) enabled the enzymatic fragmentation method to produce insert sizes similar to those obtained with Covaris shearing across all sample types and workflows (Figure 3). Specifically, the insert size difference between enzymatic and Covaris shearing ranges from 1 bp (fcDNA in the Methyl workflow) to 30 bp (FFPE DNA in the Duo workflow). Our results indicate that libraries prepared with enzymatically fragmented DNA are suitable for enrichment using the Avida target enrichment technology for a wide range of DNA samples of varying quality and quantity.

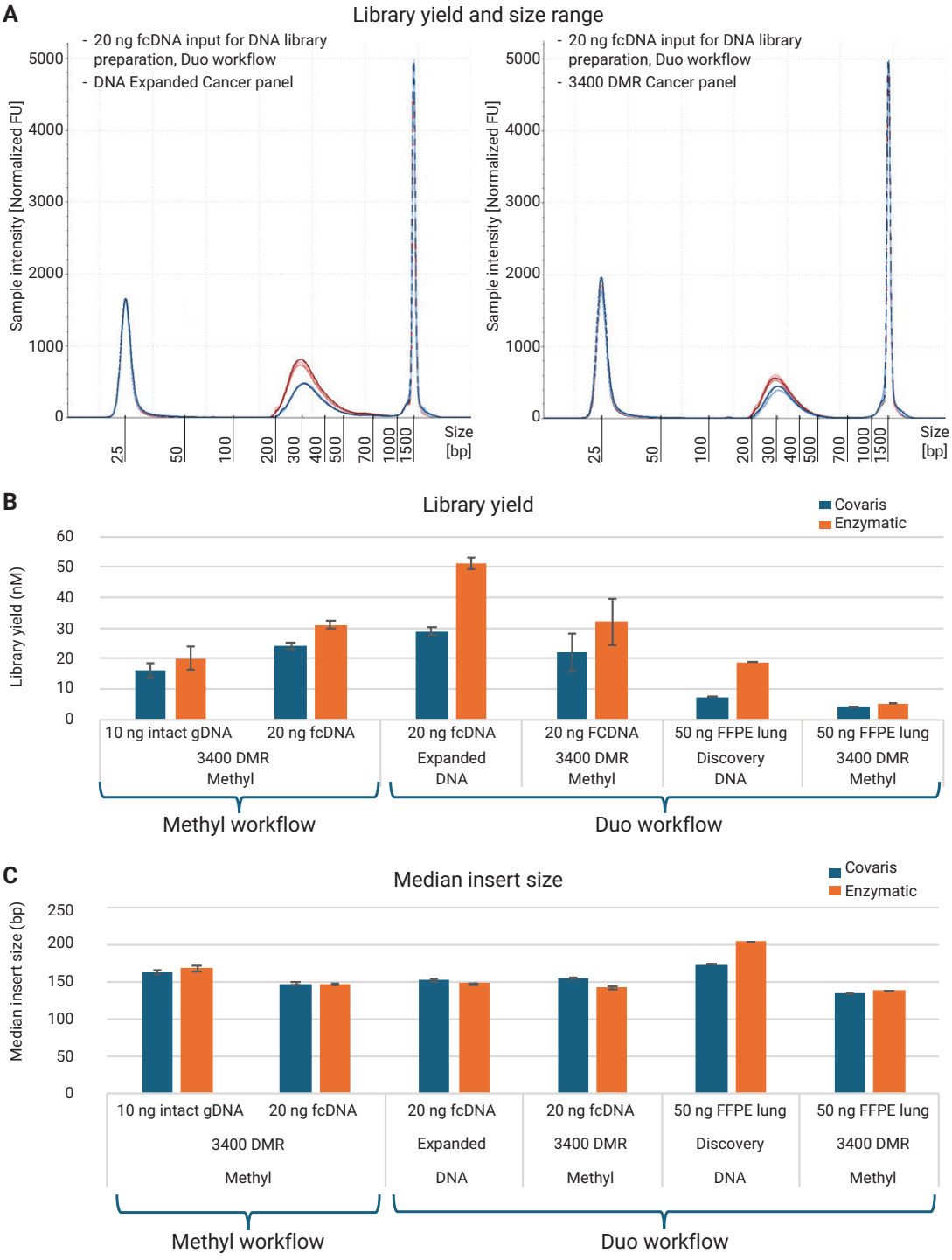

Figure 3. Comparison of library yield and insert size between enzymatic and mechanical shearing. For the Agilent Avida Methyl and Duo workflows, all samples were enriched using the Agilent Avida Methyl 3400 DMR Cancer panel. In the DNA portion of the Avida Duo workflow, 20 ng of fcDNA was enriched using the Agilent Avida DNA Expanded Cancer panel, and 50 ng of FFPE DNA was enriched using the Agilent Avida DNA Discovery Cancer panel. The Avida DNA Expanded, DNA Discovery, and Methyl 3400 DMR Cancer panels were downsampled to 25 M, 40 M, and 10 M read pairs, respectively. (A) Library yields and size ranges were quantified by the Agilent 4200 TapeStation system and Agilent D1000 ScreenTape assay, with representative electropherograms shown. (B) Quantification of library yields shows increased yields for enzymatic shearing compared to mechanical shearing. (C) Quantification of the library insert sizes shows similar insert sizes for enzymatic shearing and mechanical shearing. Each data point represents the average yield from three technical replicates, with error bars indicating standard deviations. Due to the limited DNA availability, data for the 50 ng FFPE sample were based on a single data point.

Molecule recovery, on-target rate, uniformity, and AT/GC bias

Molecule recovery, calculated as the number of unique molecules that contain a certain base of interest in the target region, was evaluated across all samples and enrichment workflows (Figure 4A). Enzymatic fragmentation followed by Avida library preparation (Methyl and Duo) consistently recovered more molecules, ranging from 1.1- to 1.5-fold higher, across all samples, panels, and enrichment workflows tested, compared to Covaris shearing.

Both shearing methods generated similar on-target rates across all samples with different panels and workflows (Figure 4B). The on-target rate is defined as the number of reads that map within the 100 bases flanking the target region. Samples enriched by the Avida DNA Expanded Cancer panel show a lower on-target rate compared to the other panels due to its relatively high percentage (~ 30%) of intronic regions for fusion detection. These intronic sequences are less complex and more repetitive, making the probes targeting them more susceptible to cross-hybridization.

Coverage uniformity is measured by the fraction of targeted regions with coverage greater than 50% of the mean coverage across all regions (Figure 4C). Both shearing methods exhibit similar coverage uniformity regardless of sample type, input amount, or workflow. Uniformity of coverage as a function of the fraction of GC content was plotted in Figure 4D for a representative panel, the Avida DNA Expanded Cancer panel. This panel targets 105 cancer-associated genes, providing either exon or hotspot coverage. Additionally, 11 of these genes include key intronic regions for detecting translocations. The data reveal that both shearing methods display even coverage across the range of GC content, from 20 to 90%, with enzymatic shearing generating slightly higher read counts.

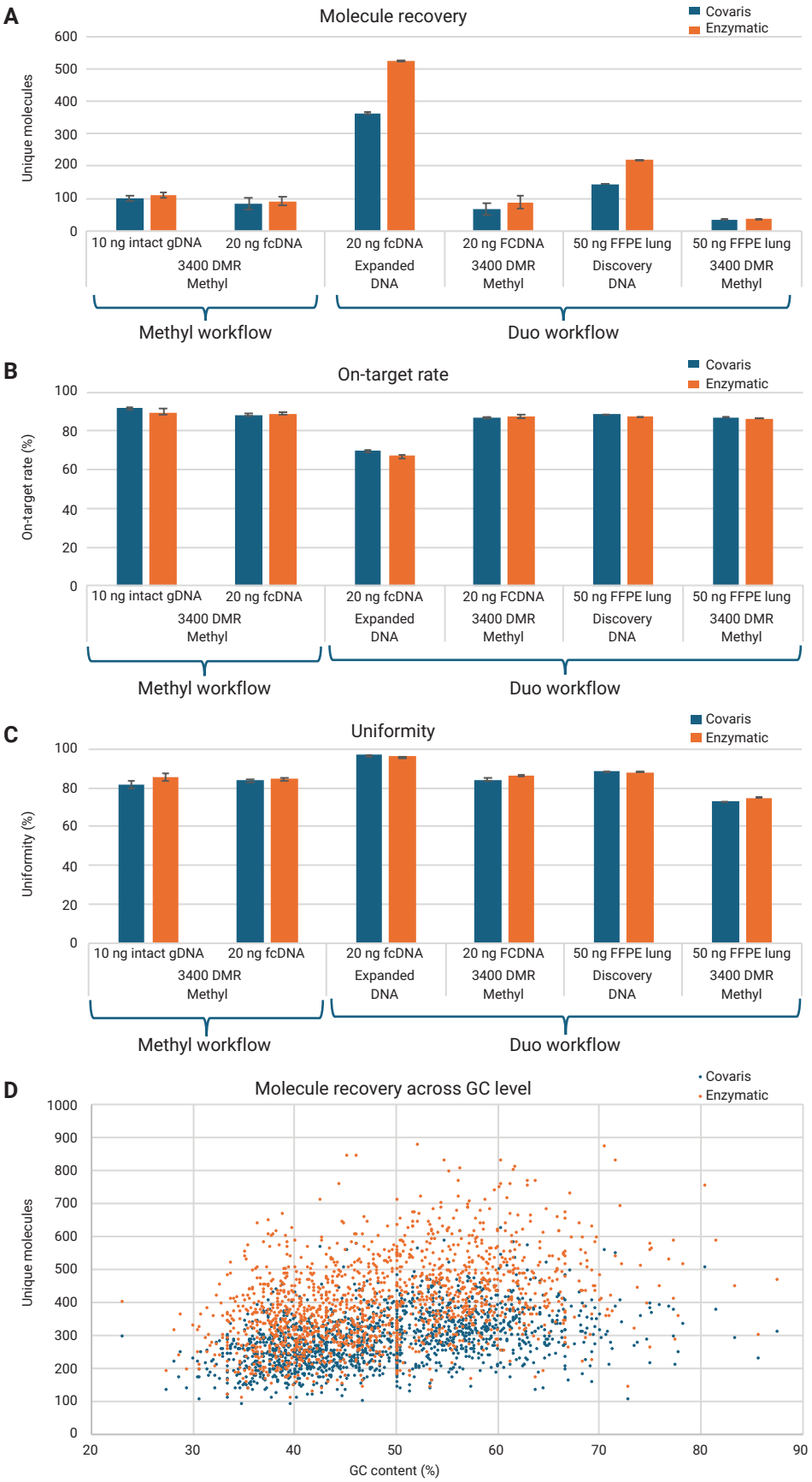

Figure 4. (A) Molecule recovery, (B) on-target rate, and (C) uniformity were across all sample types and workflows. (D) Comparable AT/GC coverage was observed at each target level in 10 ng intact gDNA samples enriched with the Agilent Avida DNA Expanded Cancer panel using the Agilent Avida Duo workflow. Agilent Avida Expanded, Discover, and Methyl 3400 DMR Cancer panels were down sampled to 25 M, 40 M, and 10 M read pairs, respectively. Each data point represents the average yield from three technical replicates with error bars indicating standard deviations, except for the FFPE samples where the data were obtained from a single replicate.

Variant detection

The Avida Duo reagent kit is optimized to detect low-frequency alleles from low-input samples. To examine the detection rate of variants at different allele frequencies (VAF) ranging from 25.5% to as low as 1%, we used an fc reference standard (HD799) and a previously characterized FFPE lung sample. The fcDNA reference HD799 was enriched with the Avida DNA Expanded Cancer panel, while the FFPE lung sample was enriched using the Avida DNA Discovery Cancer panel. All 11 variants in the fc reference standard were detected at similar rates, from samples sheared by both Covaris and enzymatic methods, aligning well with the expected VAF provided by Horizon Discovery (Table 1, Figure 5).

For the FFPE lung sample (50 ng input), the observed VAF for the G12D variant in the KRAS gene matched the expected VAF (Table 2), consistent with results from an orthogonal method demonstrating an allele frequency of 25.5% for the to the KRAS G12D variant (unpublished data). This variant was detected in samples sheared by both methods (Covaris: 32.8% VAF and enzymatic: 26.5% VAF).

Enzymatic shearing resulted in a higher number of total reads as well as reads containing the specific variants across all samples when compared to Covaris fragmentation. This outcome aligns with the higher yield and molecule recovery observed for enzymatic shearing, as illustrated in Figures 3 and 4A. These findings imply that enzymatic shearing could facilitate variant detection at a lower sequencing depth than Covaris fragmentation, potentially reducing sequencing costs.

Table 1. Variant allele frequency (VAF) detection with 20 ng fc reference standard (HD799). Data shown are the averages across three replicates. Samples were enriched with the Agilent Avida DNA Expanded Cancer panel and downsampled to 25 M read pairs (50 M total reads).

Gene	Variant	Expected VAF (%)	Covaris Shearing			Enzymatic Shearing		
			Reads containing variant	Total reads	Observed VAF (%)	Reads containing variant	Total reads	Observed VAF (%)
EGFR	T790M	1.00	21	2447	0.86	27	3620	0.75
EGFR	ΔE746 - A750	2.00	27	2065	1.31	48	3420	1.40
EGFR	L858R	3.00	96	2307	4.16	91	3529	2.58
KRAS	G12D	6.00	37	923	4.01	48	1313	3.66
PIK3CA	E545K	9.00	103	1518	6.79	140	2228	6.28
KIT	D816V	10.00	85	1110	7.66	105	1721	6.10
BRAF	V600E	10.50	209	2047	10.21	439	2899	15.14
NRAS	Q61K	12.50	121	1540	7.86	193	2097	9.20
KRAS	G13D	15.00	113	927	12.19	154	1338	11.51
PIK3CA	H1047R	17.50	316	1625	19.45	435	2076	20.95
EGFR	G719S	24.50	277	1404	19.73	594	2422	24.53

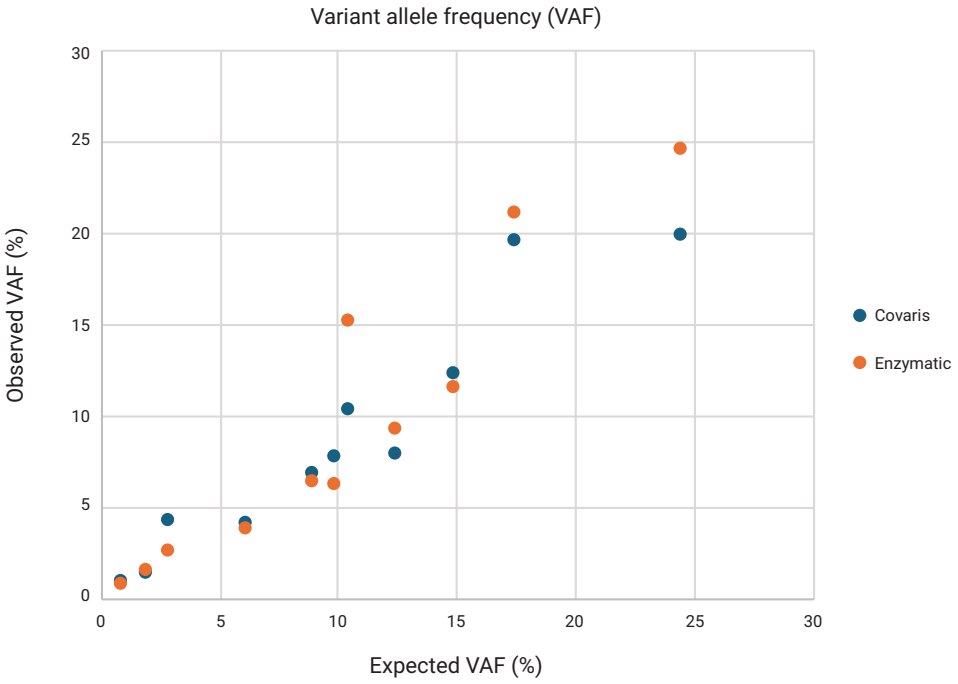

Figure 5. Good correlation between observed and expected VAF (%) using 11 variants found in the fc reference standard (20 ng HD799). The samples were fragmented using either Covaris or enzymatic shearing followed by enrichment with the Agilent Avida DNA Expanded Cancer panel in the Agilent Avida Duo workflow.

Table 2. Variant allele frequency (VAF) detection with 50 ng input of FFPE lung DNA sample. Data shown is from a single replicate. Samples were enriched with the Agilent Avida DNA Discovery Cancer panel and downsampled to 40 M read pairs (80 M total reads).

Gene	Variant	Expected VAF (%)	Covaris Shearing			Enzymatic Shearing		
			Reads containing variant	Total reads	Observed VAF (%)	Reads containing variant	Total reads	Observed VAF (%)
KRAS	G12D	25.5	44	134	32.80	57	213	26.50

Methylation assessment

Given that enzymatic fragmentation methods can potentially lead to the loss of methylation markers (unpublished data), we aimed to determine whether methylation patterns remain intact after enzymatic shearing with NEBNext UltraShear compared to Covaris shearing.

During Methyl-seq analysis, conversion treatment substitutes unmethylated cytosines with thymidine, while methylated cytosines at CpG dinucleotides sites remain unconverted. Therefore, the CpG detection rate serves as an indicator for methylated CpG presence throughout the workflow, and a decrease in this rate would suggest a loss of methylation markers during fragmentation. The CpG detection rate is expected to be similar across fragmentation methods.

We used the Avida Methyl 3400 DMR Cancer panel to determine the detection rate of methylated CpG sites across different sample types (10 ng intact gDNA, 20 ng fcDNA, and 50 ng FFPE DNA) and workflows (Methyl and Duo).

The fraction of methylated cytosines after Avida soft conversion is similar between both shearing methods and across workflows (Figure 6A). This metric measures the percentage of cytosines in the CpG context that remain unconverted (for example, methylated) to thymidine during the conversion treatment process, considering only on-target reads.

The observed variation in CpG detection rate in the fcDNA HD799 libraries is likely due to the nature of the HD799 sample. Although methylation information for this sample is not publicly available, we hypothesize that the cell lines used to create this reference standard may be hypermethylated. Furthermore, the Avida Methyl 3400 DMR Cancer panel specifically targets regions known to be hypermethylated in tumoral content. These two factors combined may have contributed to the higher percentage of methylated CpG observed in this sample compared to others.

We observed a high correlation of methylation levels between the two shearing methods using fcDNA in both Methyl and Duo workflows (Figure 6B). The methylation level refers to the proportion of cytosines that are methylated at each DMR. Both shearing methods effectively maintain methylation patterns, suggesting that enzymatic shearing is a viable alternative to mechanical shearing for preserving methylation markers, which is crucial for accurate methylation analysis.

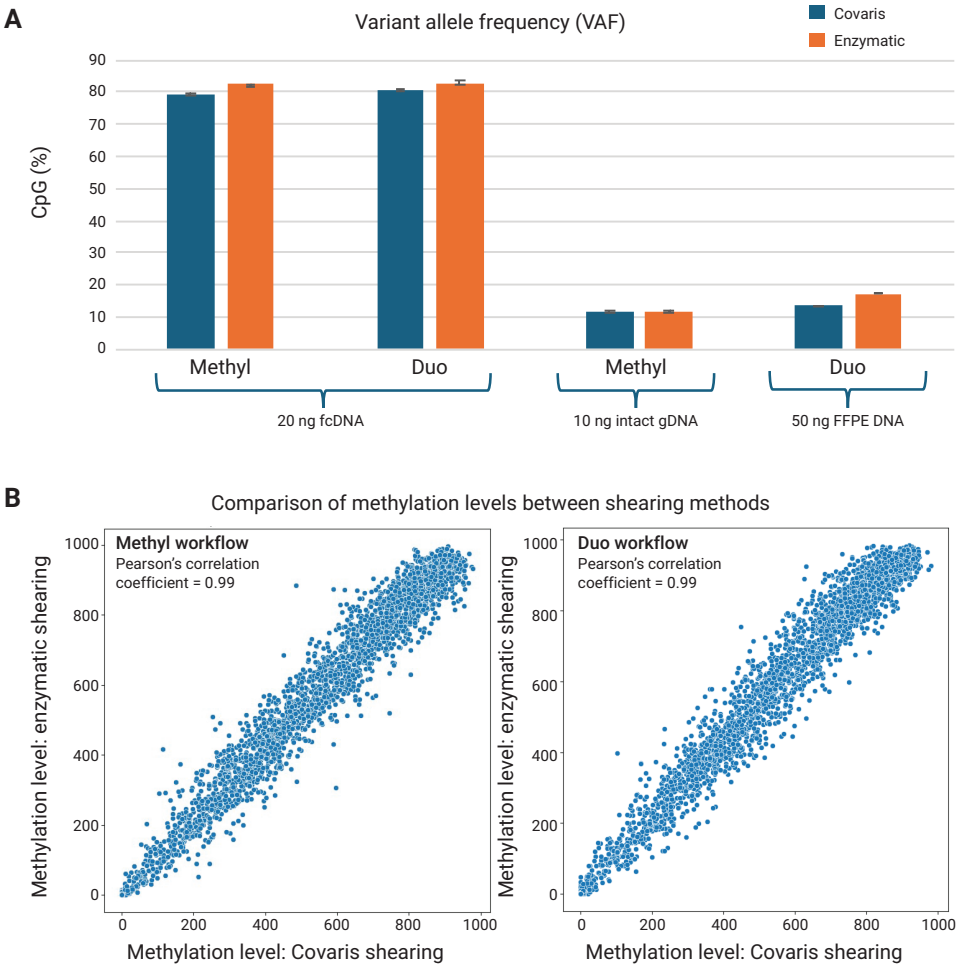

Figure 6. Similar methylation performance between samples sheared by Covaris and ensymatic fragmentation. (A) Libraries were prepared from fcDNA, intact gDNA, and FFPE lung DNA using the indicated shearing methods and Avida workflows. The fraction of methylated CpG was measured for each condition. (B) Methylation levels were plotted between Covaris and enzymatic shearing for the 20 ng fcDNA. All samples were enriched using the Agilent Avida 3400 DMR Cancer panel and downsampled to 10 M read pairs for data analysis.

Conclusion

The Avida Methyl and Duo workflows are designed to maximize information obtained from the limited material typically found in circulating tumor DNA and FFPE samples. For intact and FFPE samples requiring fragmentation upstream of library preparation, we have incorporated the NEB UltraShear enzymatic fragmentation into the Avida workflows to demonstrate that methylation markers are preserved similarly to mechanical shearing. Our results also show that enzymatic fragmentation generates slightly higher library yields and molecule recovery, while maintaining similar median fragment size, on-target rates, uniformity, SNP detection rates, and GC coverage, compared to mechanical shearing. The Agilent Avida technology offers a simple, rapid, and streamlined process to evaluate both DNA and methylation changes. Demonstrating an enzymatic shearing method that retains methylation profiles upstream of library preparation provides researchers with more flexibility and compatibility for use in automated workflows.

References

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2. Ruvolo, M. Enhancing Targeted Next-Generation Sequencing with Cooperative Hybrid-Capture for DNA and Methyl Sequencing. *Agilent Technologies Application Note* **2024**, publication number, 5594-7815EN.
3. Hsue, B.; Chen, K., T. J.; Shiryaev, S., Durand, C., and Arezi, B. A Comparative Analysis of DNA Shearing Methods Upstream of Avida DNA Workflow. *Agilent Technologies Application Note* **2025**, publication number, 5594-8275EN.
4. Costello, M.; Pugh, T. J.; Fennell, T. J.; Stewart, C.; Lichtenstein, L.; Meldrim, J. C.; Fostel, J. L.; Friedrich, D. C.; Perrin, D.; Dionne, D.; et al. Discovery and Characterization of Artifactual Mutations in Deep Coverage Targeted Capture Sequencing Data Due to Oxidative DNA Damage During Sample Preparation. *Nucleic Acids Research* **2013**, *41* (6), e67–e67. https://doi.org/10.1093/nar/gks1443.

Supplemental materials

S1. Avida DNA Targeted Sequencing Analysis Technical Guide, *Agilent Technologies user guide*, **2024**, publication number G9409-90001. https://www.agilent.com/cs/library/usermanuals/public/G9409-90001.pdf

S2. Avida Targeted Methylation Sequencing Analysis Technical Guide, *Agilent Technologies user guide*, **2025**, publication number G9419-90001. https://www.agilent.com/cs/library/usermanuals/public/G9419-90001.pdf

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