Demonstrating Analytical Performance of Agilent Avida Duo Utilizing Novel Multiomic Reference Standards

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Introduction

Dual interrogation of somatic mutations and differentially methylated regions (DMRs) in cell-free samples (cfDNA) is a powerful approach in oncology research. Conventional NGS target enrichment workflows accomplish this by splitting a sample into two tests, one for mutation and one for DMR methylation measurement. This sample-splitting reduces the input for each test, potentially limiting sensitivity. The Agilent Avida target enrichment technology allows enrichment of native DNA fragments, preserving epigenetic methylation marks while also allowing for mutation detection. This method, termed Avida Duo, maximizes the information from a single cfDNA sample input (Figure 1).

Currently, standards with known variants or methylation levels are available but there is a lack of multiomic standards for evaluation of advanced dual modalities. Therefore, we utilized commercially available Seraseq mutation and methylation standards to create custom reference blends to assess analytical performance of the Avida Duo workflow. The references were fully characterized then combined in defined ratios to achieve variant allele frequencies and methylation levels representative of real-world cfDNA samples. Analytical performance of Avida Duo workflow was assessed on these custom references with a 55.2 kb mutational panel and a 173.9 kb methyl panel covering biologically relevant DMRs. Here we demonstrate robust detection of variants down to 0.5% variant allele frequencies and 0.5% DMR methylation from 5 and 10 ng inputs, with Avida Duo multiomic workflow.

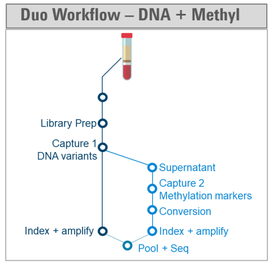


Figure 1. Avida Duo Workflow. Library preparation takes 2 hours to generate adapter-ligated native cfDNA that retains methylation marks at CpGs, proceeding directly to Capture 1, using an Avida DNA variant Panel. The same library is used for Capture 2, using an Avida Methyl Panel targeting methylation markers. The Avida Duo workflow takes 8½ hours to obtain enriched libraries ready for sequencing.

Experimental

Seraseq ctDNA v4 AF5% (0710-3100), Unmethylated ctDNA (0710-3088) and Methylated ctDNA (0710-3089) were purchased from LGC Clinical Diagnostics. To achieve known variant allele frequencies (VAF) and methylation levels (METH), ctDNA v4 and Unmethylated references were combined to prepare blends at 1% VAF, 1% METH and 0.5% VAF, 0.5% METH combinations. Customized reference samples thus prepared were tested at 10 ng and 5 ng inputs.

Libraries were prepared using the Agilent Avida Duo Methyl reagent kit. For the DNA part of the Avida Duo workflow, a 55.2 kb Avida DNA Custom panel targeting 65 known variants present in the ctDNA v4 reference, was designed and employed. Enriched DNA libraries were sequenced at 65,000X depth on NovaSeq6000. DNA data was processed by fgbio (version 2.1.0) single consensus UMI deduplication filtered for 2 minimum reads, followed by variant calling using Vardict (version 1.8.3).

For the Methyl part of the Avida Duo workflow, a 173.9 kb Avida Methyl Custom panel targeting cancer-specific DMRs, was designed and employed. Enriched Methyl libraries were sequenced at 1,500X depth. Methylation Index (MI) score was computed on methylation data.

Results and Discussion

Characterization of Seraseq references with DNA and Methyl panels

Prior to preparing custom blends, the methylation profile of the mutation-specific reference was determined, and the wild-type variant status of the methylation-specific references were confirmed. Performance of the Avida DNA and Methyl panels were assessed on references and custom blends.

Results and Discussion

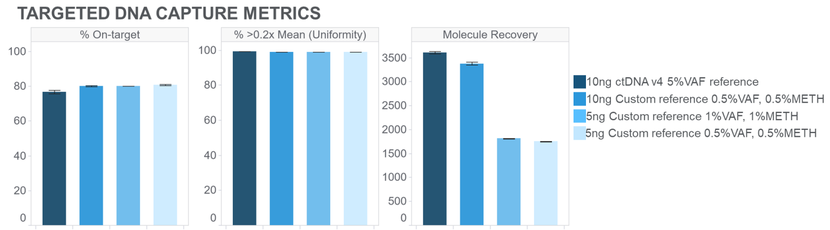


Figure 2. Capture performance of the Avida Duo workflow performed with the DNA panel. High >75% On-target, high % >0.2x Mean coverage reflective of uniform enrichment of target regions, and high Molecule Recovery was observed for ctDNA v4 reference and customized reference blends.

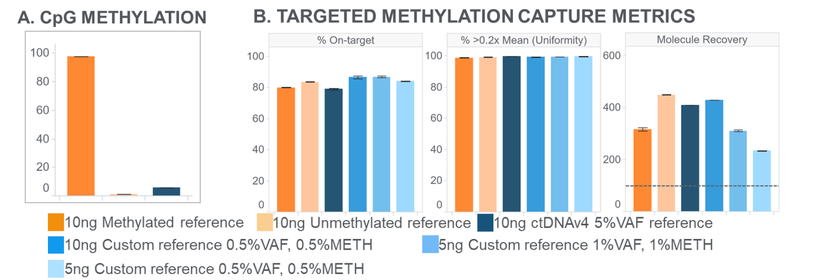


Figure 3. Methyl enrichment metrics using the methyl panel with the Avida Duo workflow. **A)** The expected methylation levels were observed for the 100% and 0% methylation standards. The ctDNA v4 standard showed ~6% methylation. **B)** Key target capture metrics. High >75% On-target, high % >0.2x Mean coverage (uniformity) was observed for references and customized reference blends. Molecule recovery was consistently above the empirically determined threshold (shown by dashed line) needed for robust measurement of target DMR CpG methylation.

Conversion efficiency and coverage over target GC

The Avida Duo workflow was compared to a commercially available, targeted methylation system that employs enzymatic conversion chemistry. Robust conversion of 99.4% non-methylated cytosines was observed for Avida soft conversion treatment. A uniform coverage over target DMRs, by GC fraction, was observed for the Avida Duo workflow.

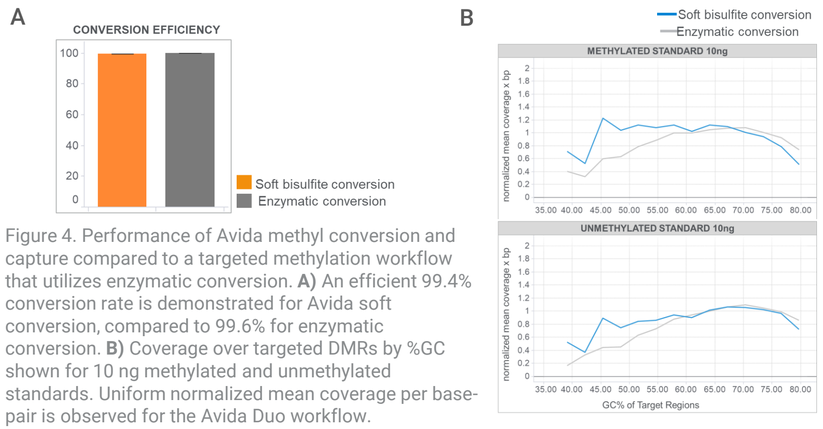


Figure 4. Performance of Avida methyl conversion and capture compared to a targeted methylation workflow that utilizes enzymatic conversion. **A)** An efficient 99.4% conversion rate is demonstrated for Avida soft conversion, compared to 99.6% for enzymatic conversion. **B)** Coverage over targeted DMRs by %GC shown for 10 ng methylated and unmethylated standards. Uniform normalized mean coverage per base-pair is observed for the Avida Duo workflow.

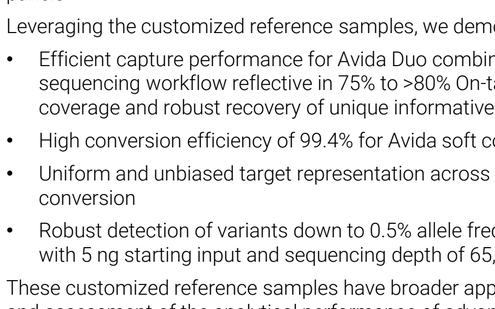
Results and Discussion

Assessment of Avida Duo performance using customized reference samples

A. Duo DNA

Input	Custom Reference (Expected)		Variant Detection	
	%VAF	%METH	Sensitivity TP/(TP+FN)	Specificity TN/(TN+FP)
10 ng	0.5%	0.5%	98% (64)	98%
5 ng	1%	1%	98% (64)	99%
	0.5%	0.5%	94% (61)	99%

B. Read Depth and Allele frequency (AF)



C. Duo Methyl

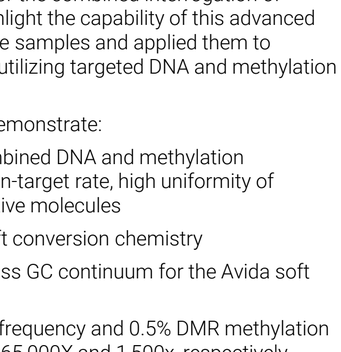


Figure 5. Highly sensitive detection of DNA variants and DMR methylation using the Avida Duo workflow. **A)** Variant detection showed ≥94% sensitivity and ≥98% specificity at 1% VAF and 0.5% VAF for 10 ng and 5 ng inputs. The number of variants detected out of 65, is shown in parenthesis. **B)** The high variant calling sensitivity and specificity is enabled by the depth of coverage achieved over target regions after UMI deduplication. The measured AF is in agreement with the expected 0.5% VAF and 1% VAF (red dashed lines). **C)** Methylation Index (MI) score was determined for the Duo targeted Methyl data. MI is an Agilent proprietary methylation profiling tool which computes a methylation score that reflects the overall methylation status of a sample. MI profiling tool sensitively detects and reports scores for 1% METH and 0.5% METH expected from the customized reference samples.

Conclusions

The Avida Duo technology offers a single workflow for the combined interrogation of genetic and epigenetic information of cfDNA. To highlight the capability of this advanced multiomic assay, we developed customized reference samples and applied them to benchmark performance of the Avida Duo workflow utilizing targeted DNA and methylation panels.

Leveraging the customized reference samples, we demonstrate:

- Efficient capture performance for Avida Duo combined DNA and methylation sequencing workflow reflective in 75% to >80% On-target rate, high uniformity of coverage and robust recovery of unique informative molecules
- High conversion efficiency of 99.4% for Avida soft conversion chemistry
- Uniform and unbiased target representation across GC continuum for the Avida soft conversion
- Robust detection of variants down to 0.5% allele frequency and 0.5% DMR methylation with 5 ng starting input and sequencing depth of 65,000X and 1,500x, respectively

These customized reference samples have broader applicability for assay development, QC, and assessment of the analytical performance of advanced multiomic assays like the Avida Duo workflow.