Bravo Automation of Agilent Avida Targeted Enrichment for High-Throughput Detection of Genomic Alteration and DNA Methylation

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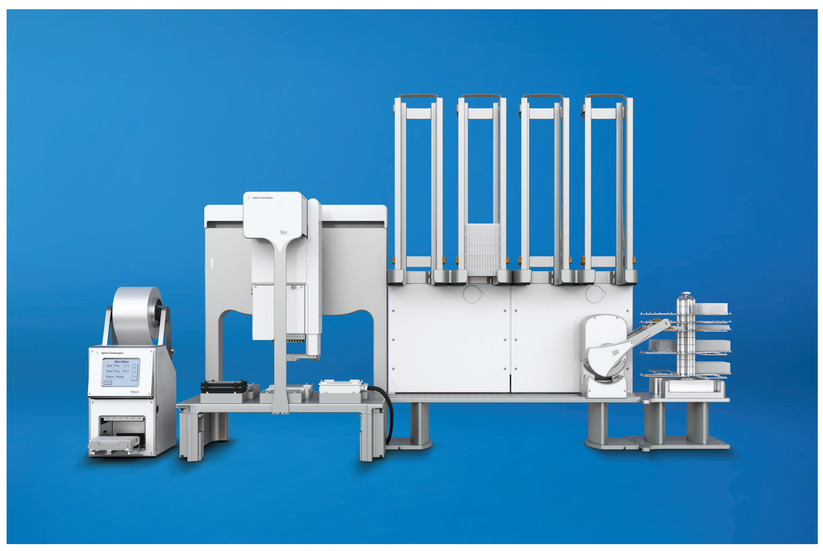
Introduction

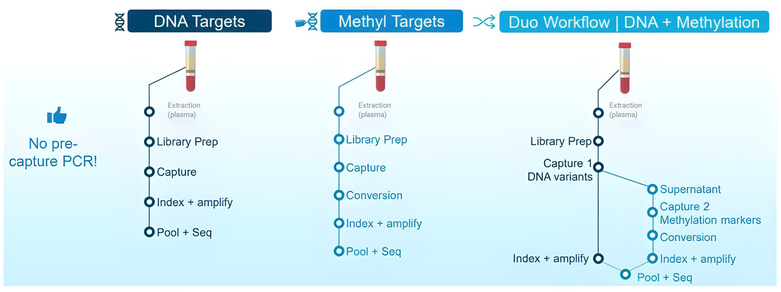
Current genomic and epigenomic profiling of cancer tissue DNA or cfDNA (cell-free DNA) in liquid biopsy relies upon separate, time- and sample-consuming technologies for somatic variant detection or methylation analysis. Here we describe workflow and performance of the Agilent Bravo automated liquid handling platform with the Agilent Avida targeted enrichment solution for somatic variant and methylation profiling. This solution can effectively analyze low-input tumor DNA or cfDNA samples. The Avida Duo workflow enables highly sensitive detection of single nucleotide variant (SNV), insertions and deletion (INDEL), copy number variation (CNV), Translocation (TL), and DNA methylation profiles from a single sample, without any sample splitting.

Experimental

Genomic DNA , FFPE and cfDNA samples were captured using Avida workflow and panels. All library preparation workflows were automated on Bravo NGS workstation (see below).

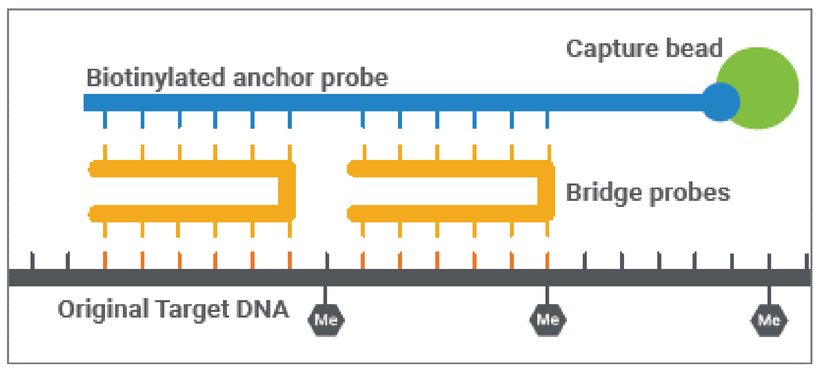


Workflow schematic for Avida DNA, Methyl and Duo



Single day workflows | Two-day workflow

Capture Methodology



All Bravo automated samples were sequenced as 2x150 paired end reads (Illumina). Samples were aligned with bwa.mem. Methyl seq samples were aligned with Bismark. Sequencing depths were tailored to input levels, panels and variant detection requirements (duplication rate target ≥ 80%). Variant detection and methylation index were determined using Alissa Reporter (Agilent, Inc). Ultra-low allele frequency variants in cfDNA samples were identified using VarDict (version 1.5.0) and filtered with an internal analysis pipeline. CNVs were analyzed using CNVkit (version 0.9.8). Translocation analysis was performed using GeneFuse software (version 0.6.1).

Results and Discussion

Performance Summary of gDNA(n=96) and cfDNA(n=8) samples captured with Focused Cancer Panel (27Kb) with Avida DNA workflow on Bravo NGS workstation

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Avida DNA Focused Cancer panel (27Kb)

Gene	Category	Coverage in 3ng cfdna (n=8)	
		Average	CV
AKT1	exons 2,3,10	1672	0.06
ALK	exons 20-29, exon 5 hotspot	1637	0.06
BRAF	Exon 2,3 Hotspot Region	1367	0.08
EGFR	Exon 1,5,6,8 Hotspot Region	1626	0.07
	exons 18-21	1607	0.06
ERBB2	exons 1-27	1690	0.06
ESR1	exons 2-9	1611	0.06
KRAS	exons 2-5	1447	0.06
KRAS_NM_004985	NM	1039	0.07
MAP2K1	Exons 2,3,6 Hotspot Region	1719	0.06
MET	Exons 2-21	1442	0.06
NRAS	Exons 2-5	1516	0.06
PIK3CA	Exons 2,3,5,6,8,9,10,19,20,21 Exon 14 Hotspot	1307	0.06
RET	Exons 6, 10-20	1597	0.06
ROS1	Exons 1-43	1417	0.06
TP53	exons 2-11	1693	0.06

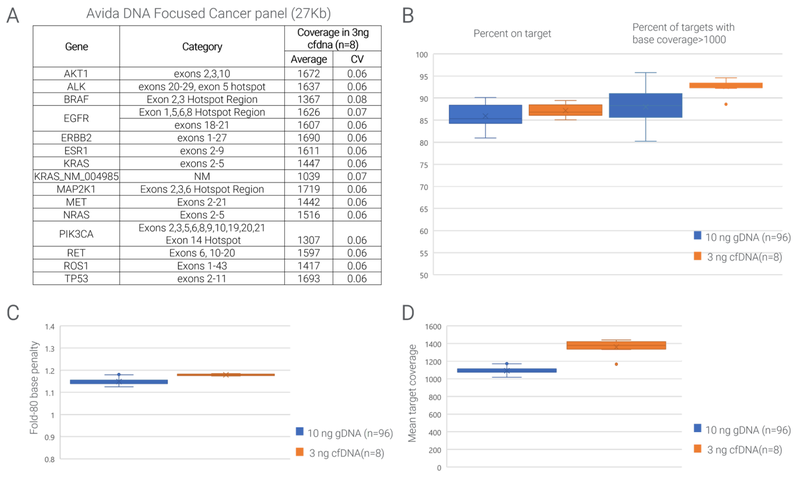

Figure1: High reproducibility across samples processed on Bravo NGS workstation. Hapmap NA24385 (Coriell, Inc) and Human cfDNA control (Biochain, Inc) were captured and analyzed at theoretical coverage of ~39,000X (A)List of targets in the Focused Cancer Panel and average coverage in cfDNA samples. (B)High percent on target and fraction of targets with base coverage >1000X (C) Uniformity measured by Fold-80 base penalty (Hsmetrics, Broad Institute)(D)High recovery of input material demonstrated by deduped mean target coverage.

Variants detected in cfDNA reference standard samples using Avida DNA workflow and Expanded cancer panel (340Kb) on Bravo NGS workstation

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Avida DNA Expanded Cancer panel (340Kb)
104 Genes with exonic/ hotspot coverage for SNP detection

ABL1	AKT1	AKT2	ALK	AR	ARAF	ARID1A	ATM	ATR	ATRX	B2M	BAP1	BARD1
BRAF	BRCA1	BRCA2	BRD4	BRIP1	CCND1	CD274	CDK12	CDK4	CDKN2A	CHEK1	CHEK2	CTNNB1
DDR2	DNMT3A	EGFR	ERBB2	ERBB3	ERBB4	ERCC2	ESR1	EZH2	FANCA	FANCC	FANCL	FBXW7
FGF3	FGFR1	FGFR2	FGFR3	FGFR4	FLT3	GNA11	GNAQ	GNAS	HGF	HRAS	IDH1	IDH2
JAK1	JAK2	KEAP1	KIT	KRAS	MAP2K1	MAP2K2	MDM2	MET	MLH1	MSH2	MSH6	MTOR
MYC	MYD88	NF1	NF2	NPM1	NRAS	NTRK1	NTRK2	NTRK3	PALB2	PDGFRA	PDGFRB	PIK3CA
PMS2	POLE	PTCH1	PTEN	PTPN11	RAC1	RAD50	RAD51B	RAD51C	RAD51D	RAD54L	RAF1	RB1
RET	RICTOR	ROS1	SMARCA4	SMARCB1	SMO	STAG2	STK11	TERT	TP53	TSC1	TSC2	VHL

13 Genes with intronic coverage for translocation detection

ALK	BRAF	EGFR	FGFR1	FGFR2	FGFR3	NRG1
NTRK1	NTRK2	NTRK3	RAF1	RET	ROS1	

B

Sample	Type of Variant	Gene	Variant	Panel	Vendor Reported %VAF	Detected %VAF (n=8)
HD786	CNV	MET	Amplification	Expanded Plus (340Kb)	4.5 copies	7 copies
	SNV	GNA11	c.626A>T		5.6	5.2
		AKT1	c.49G>A		5	4.9
		PIK3CA	c.1633G>A		5.6	5.2
		EGFR	c.2300_2308dup		5.6	4.4
		EGFR	c.2235_2249del		5	3.6
	Translocation	SLC34A/ROS1			5.6	4.5
		RET/CCDC6			5	1.3

Table 1: Variants detected in Structural Multiplex cfDNA Reference Standard HD786 (Horizon Discovery, Inc) samples using Avida DNA workflow captured with Expanded Cancer panel. All samples were analyzed at a theoretical coverage of ~22,000X.

SNVs, CNVs and Translocations detected in SeraCare V4 ctDNA (beta test material) reference sample

A

Gene	Variant	Detected %VAF using Expanded cancer panel		Detected %VAF using Focused panel	
		10ng, 0.5% Replicate1	10ng, 0.5% Replicate 2	10ng, 0.5% Replicate 1	10ng, 0.5% Replicate 2
AKT1	p.E17K	0.35	0.50	0.49	0.47
AR	p.H875Y	0.77	0.77	Not targeted	
ATM	p.C353fs	0.29	0.57		
BRAF	p.V600E	0.33	0.27	0.11	0.29
BRCA1	p.K654fs	0.61	0.59	Not targeted	
BRCA2	p.R2645fs	0.39	0.32		
CDKN2A	p.P11_S12insAAGSSMEP	0.14	0.68		
CHEK1	p.T226fs	0.48	1.00		
EGFR	p.E746_A750del	0.33	0.45	0.39	0.29
	p.S768I	0.41	0.29	0.35	0.23
	p.D770_N771insG	0.55	0.28	0.30	0.26
	p.T790M	0.62	0.45	0.51	0.43
	p.C797S	0.67	0.46	0.33	0.46
	p.L858R	0.24	0.45	0.51	0.49
ERBB2	p.A775_G776insYVMA	0.47	0.62	0.41	0.27
ESR1	p.D540G	0.39	0.39	0.70	0.35
FGFR3	p.S249C	0.41	0.20	Not targeted	
HRAS	p.Q61R	0.50	0.60		
	p.G13R	0.34	0.40		
IDH1	p.R132C	0.47	0.68		
IDH2	p.R140Q	1.02	1.01		
	p.R172K	1.18	0.92		
KIT	p.D816V	0.46	0.33		
KRAS	p.Q61H	0.43	0.98	0.30	0.55
	p.G12C	0.72	0.71	1.23	0.87
	p.G12D	0.83	0.60	1.02	1.08
MAP2K1	p.P124S	0.53	0.35	0.50	0.24
MET	p.T1024fs	0.57	0.57	0.42	0.29
MLH1	p.E78fs	0.51	0.31	Not targeted	
MSH6	p.G686fs	0.38	0.16		
MTOR	p.S2215Y	0.24	0.60		
NF1	p.F1247fs	0.87	0.77		
NRAS	p.Q61R	0.65	0.38	0.28	0.41
NTRK1	p.G595R	0.49	0.25	Not targeted	
NTRK2	p.G639R	0.65	0.66		
NTRK3	p.G623R	0.45	0.59		
PALB2	p.N280fs	0.55	0.43		
PDGFRA	p.D842V	0.52	0.14		
PIK3CA	p.E545K	0.24	0.44	0.28	0.40
	p.H1047R	0.50	0.36	0.23	0.46
	p.N1068fs	0.55	0.34	0.32	0.46
PMS2	p.R287fs	0.48	0.19	Not targeted	
PTCH1	p.TR769*	0.50	0.44		
PTEN	p.K440fs	0.74	0.49		
	p.P421fs	0.55	0.43		
RAD51C	p.S81*	0.20	0.37		
	p.G114fs	0.31	0.47		
RAF1	p.S257L	0.39	0.23		
RB1	p.R251*	0.33	0.70		
RET	p.M918T	0.39	0.44	0.19	0.64
SMARCB1	p.R40*	0.28	0.34	Not targeted	
STK11	NM_000455:c.734+1G>T	0.49	0.33		
TERT	NM_198253:c.-124C>T	0.50	0.25		
	NM_198253:c.-146C>T	0.41	0.20		
TP53	p.C242fs	0.46	0.42	0.26	0.98
	p.R248Q	0.55	0.46	0.23	1.01
	p.R273H	0.59	0.50	0.68	0.52
TSC1	NM_000368:c.1263+1G>T	0.71	0.48	Not targeted	
TSC2	NM_000548:c.2640-1G>A	0.63	0.42		
VHL	p.R161*	0.34	0.65		

B

CNV gene (Expected Fold change 1.3)	Fold change detected (n=2)
AKT2	1.27
CCND1	1.97
ERBB2	1.32
FGF3	1.43
FGFR1	1.35
MET	1.68
MYC	1.33

C

Translocation	Translocation location	0.5% 10ng	
		r1	r2
CD74::ROS1	Intron 6::Intron 34		detected
EML4::ALK	Intron 13::Intron 19	detected	detected
ETV6::NTRK3	Intron 5::Intron 14		detected
FGFR3::TACC3	Exon 18::Intron 7	detected	detected
NCOA4::RET	Intron 7::Intron 11	detected	detected
PML::NTRK2	Intron 2::Intron 12	detected	
TPM3::NTRK1	Intron 7::Intron 9	detected	detected
	Detection rate	71%	86%

Table 2: Low allele frequency variants at 0.5% VAF were detected in 10ng input SeraCare V4 ctDNA (LGC, Inc) captured with Focused Cancer Panel or Expanded Cancer Panel using Avida DNA workflows. Expanded cancer panel covers 60 SNP/INDELs (0.5% VAF), 7 translocations (0.5% VAF) and 7 CNVs (1.3-fold amplification) in the SeraCare V4 ctDNA sample. Theoretical coverages of ~222,000X and ~44,000X were used for variant detection with Focused Cancer panel and Expanded Cancer panel respectively.(A) SNV allele frequencies detected in SeraCare V4 ctDNA (LGC, Inc) (B) CNVs detected with Avida DNA Expanded Cancer Panel. (C) Translocations detected with Avida DNA Expanded Cancer Panel.

Results and Discussion

Combined detection of variants and DMRs from paired cancer FFPE/cfDNA samples captured using Expanded Cancer Panel and Methyl 3400 DMR Cancer Panel using Avida Duo workflow

A

Case number	Cancer type	Overal Clinical Stage	Tumor fraction in FFPE	DIN value NAT	DIN value FFPE	Variants	Classification	Vendor reported % VAF in FFPE	Detected % VAF in FFPE	Detected % VAF in cfDNA (Derived from 1ml plasma)
1	Colorectal Cancer, Adenocarcinoma	II-A	50%	N/A	2.8	KRAS, c.1397 G>A TP53, c.524G>A	Pathogenic Pathogenic	20 18	15.2 25.4	0.69 1.00
2	Colorectal Cancer, Adenocarcinoma	II-A	70%	N/A	2.5	CTNNB1, c.110C>T EZH2, c.2239G>T MSH2,c.1738G>T PIK3CA, c.1624G>A, PIK3CA, c.1070G>A PTEN, c.389G>A	Pathogenic Likely Pathogenic Pathogenic Pathogenic Pathogenic Pathogenic	27 26 30 22 23 55	17.5 16.2 13.8 12.7 10.6 30	ND 0.09 0.07 ND 0.05 0.04
3	NSCLC	III-B	100%	5.4	4.8	TP53, c.464C>T SMARCB1, c.478A>T (Based on tumor-normal comparison)	VUS Stopgain	N/A	5 7.9	0.04 0.08

B

Case number	Cancer type	Overal Clinical Stage	Tumor fraction in FFPE	DIN value NAT	DIN value FFPE	Methylation index score in NAT	Methylation index score in FFPE	Methylation index score in cfDNA
1	Colorectal Cancer, Adenocarcinoma	II-A	50%	N/A	2.8	N/A	59.485	2.122
2	Colorectal Cancer, Adenocarcinoma	II-A	70%	N/A	2.5	N/A	65.108	0.328
3	NSCLC	III-B	100%	5.4	4.8	1.352	8.155	0.856
	Control cfDNA (n=4)	N/A	0%	N/A	N/A	N/A	N/A	0.206

Table 3: Variants and DMR (differentially methylated regions/Methylation index score) detected from normal tissue adjacent to tumor (NAT), tumor FFPE and cfDNA samples. All samples were captured using the Avida DNA Expanded Cancer panel followed by the Methyl 3400 DMR Cancer panel (876Kb) using the Avida Duo workflow. Tumor samples were analyzed at ~22,000X theoretical coverage and cfDNA samples were analyzed at ~100,000X for variant detection. All tumor and cfDNA samples were analyzed at ~3400X for DMR detection.(A) SNVs detected in tumor FFPE were also detected in matched cfDNA samples (B) Methylation index scores in NAT, Tumor FFPE and cfDNA captured with Methyl 3400 DMR Cancer Panel. ND=Not detected

Conclusion

- Avida workflow on Bravo, at 96 sample capacity, exhibits highly reproducible on target performance, uniformity, and efficient molecule recovery (~75% for 3ng cfDNA)
- Single day turnaround for Avida DNA (5-7 hrs) or Methyl (6-8hrs) workflows.
- SeraCare V4 ctDNA reference captured with the Avida DNA Expanded Cancer Panel showed 100% detection for 60 SNVs/Indel (0.5% VAF), 100% detection for 7 CNVs (1.3-fold amplification) and 71-86% detection for translocations (0.5% VAF).
- FFPE and cfDNA samples from stage II/III cancers processed with Avida Duo workflow showed:
  - Variants identified in tumor FFPE sample were also detected in cfDNA samples.
  - FFPE and cfDNA samples from cancer patient samples exhibited higher methylation scores compared to controls.
- Avida Duo workflows allow combined analysis of low frequency variant allele detection and methylation with as little as 3ng cfDNA