Techniques for Measuring Cancer Burden in Liquid Biopsy Samples

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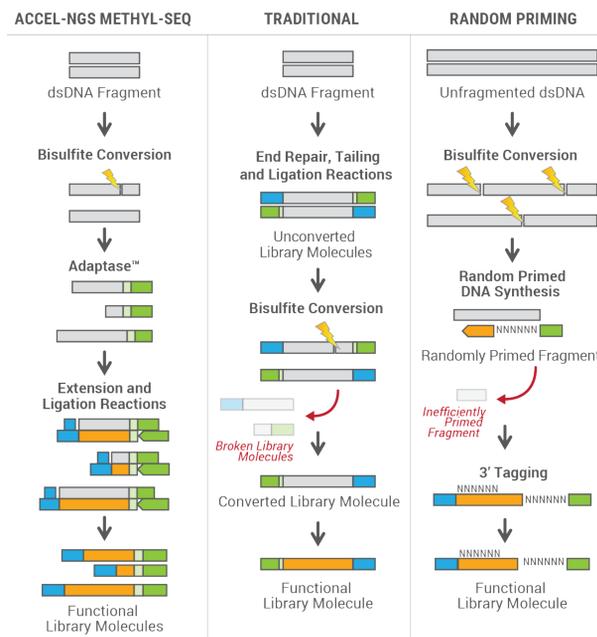
Abstract

Liquid biopsy is a non-invasive sample source that can be utilized to assess cancer burden by measuring the tumor-derived fraction of circulating, cell-free DNA (cfDNA) from plasma. We evaluated two assays to monitor cancer burden using cfDNA: whole genome bisulfite sequencing (WGBS) and targeted amplicon sequencing for 56 oncology-related genes. We tested samples with both assays to characterize their efficacy across a broad spectrum of cancer types, stages, and treatment regimens. cfDNA was extracted from tumor-bearing patients and normal controls. To monitor methylation density, WGBS was performed using 5 ng of bisulfite-converted cfDNA with the Accel-NGS® Methyl-Seq DNA Library Kit. To detect tumor-specific mutations, 10 ng of cfDNA was used for the Accel-Amplicon™ 56G Oncology Panel. Six out of eight cancer samples demonstrated significant hypomethylation in cfDNA, ranging from 2-40% when compared to healthy controls. The 56 gene amplicon panel identified point mutations in the cfDNA of only three samples, but which also had the highest observed hypomethylation (18-40%). For all but two cancer samples, corresponding mutations were also found in the primary tumor at allele frequencies significantly higher than in the cfDNA fraction (e.g., 22% in tumor vs. 5% in cfDNA). The three cancer samples that had primary tumor mutations that were not detected in cfDNA also had the lowest observed hypomethylation. Therefore, a correlation between hypomethylation and detection of tumor mutations in the cfDNA fraction may exist. Further studies will elucidate which assay is more sensitive at detecting tumor burden in cfDNA.
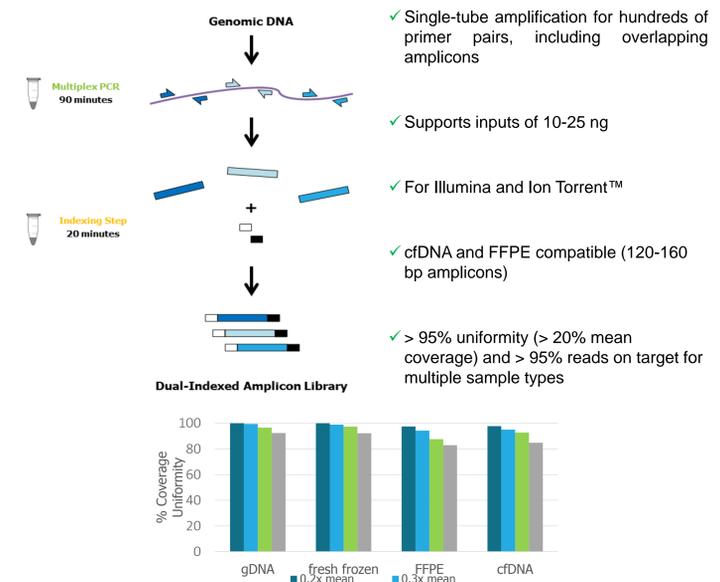
Experimental Design

Tumor bearing blood, n = 8 (Streck Cell-Free DNA BCT®)

Healthy control blood, n = 5 (Streck Cell-Free DNA BCT)

Isolate cfDNA with QIAamp® Circulating Nucleic Acid kit

5 ng · 10 ng

Accel-NGS Methyl-Seq	Accel-Amplicon 56G Oncology Panel
Bisulfite conversion and Accel-NGS Methyl-Seq library construction	Accel-Amplicon 56G Oncology Panel library construction
Calculate hypomethylation status of cancer samples compared to healthy controls from 10M Illumina® MiSeq® reads using Methypipe.	LoFreq and GATK variant calling from 5000X coverage was compared to corresponding tumor FFPE and normal adjacent tissues.

Accel-NGS Methyl-Seq Kit

Bisulfite-Treated ssDNA Fragments

- Adaptase™ 17 min
- Extension + SPRI 23 min
- Ligation + SPRI 30 min
- Indexing PCR + SPRI

Library

- ✓ 2-hour library prep workflow
- ✓ Post-bisulfite prep uses efficient Adaptase ssDNA library technology
- ✓ Supports input range of 100 pg to 100 ng with minimal PCR cycles
- ✓ Retains high sequence complexity for comprehensive and uniform methylome coverage

Accel-NGS 2S Hyb Kit

Fragmented dsDNA

- Repair I — Dephosphorylation 27 min
- Repair II — End Repair & Polishing 32 min
- Ligation I — 3' Ligation of P7 27 min
- Ligation II — 5' Ligation P5 22 min
- Pre-Hyb PCR — varies
- Hybridization Capture
- Post-Hyb PCR

Sequencing

- ✓ Simple with-bead protocol
- ✓ Broad input range 10 pg - 1 µg
- ✓ Repairs both 5' and 3' termini to enhance ligation efficiency
- ✓ Compatible with cfDNA and FFPE samples
- ✓ Increased library complexity
- ✓ Balanced coverage of AT-/GC-rich regions

Superior Methyl-Seq Performance

ACCEL-NGS METHYL-SEQ | TRADITIONAL | RANDOM PRIMING

1 ng Arabidopsis DNA Input	Swift Methyl-Seq	Traditional	Random Priming
% Reads Aligned	83.3%	80.7%	73.4%
Avg. Genome Coverage	18X	10X	12X
% Genome Covered ≥ 10x	77.0%	17.0%	31.0%
% Duplicate Reads	18.0%	62.0%	46.0%
Estimated Library Size	38 M	6 M	12 M

Paired-end sequencing was performed on a HiSeq® with V4 chemistry with 125 bp PE. Analysis performed using BSMap and Picard tools using 30M reads for each method for direct comparison.
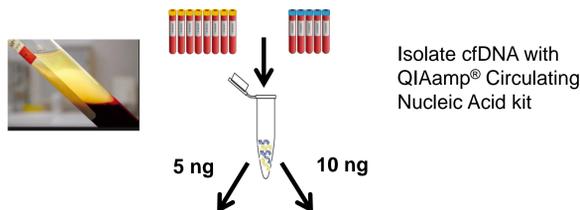
Accel-Amplicon 56G Oncology Panel

Genomic DNA → Multiplex PCR 90 minutes → Indexing Step 20 minutes → Dual-Indexed Amplicon Library

- ✓ Single-tube amplification for hundreds of primer pairs, including overlapping amplicons
- ✓ Supports inputs of 10-25 ng
- ✓ For Illumina and Ion Torrent™
- ✓ cfDNA and FFPE compatible (120-160 bp amplicons)
- ✓ > 95% uniformity (> 20% mean coverage) and > 95% reads on target for multiple sample types

56G Panel: Hotspot and Comprehensive Coverage of 56 Oncology-Related Genes

Gene	#	Gene	#	Gene	#	Gene	#	Gene	#	Gene	#
ABL1	5	CSF1R	2	FBXW7	6	GNAS	2	KIT	14	NPM1	1	STK11	5
AKT1	2	CTNNB1	2	FGFR1	2	HNF1A	4	KRAS	3	NRAS	3	SMAD4	10
ALK	2	DDR2	1	FGFR2	4	HRAS	2	MAP2K1	5	PDGFRA	4	SMARCB1	4
APC	9	DNMT3A	4	FGFR3	6	IDH1	1	MET	6	PIK3CA	11	SMO	5
ATM	19	EGFR	9	FLT3	4	IDH2	1	MLH1	1	PTEN	14	SRC	1
BRAF	4	ERBB2	6	FOXL2	1	JAK2	1	MPL	1	PTPN11	3	TP53	21
CDH1	4	ERBB4	8	GNA11	2	JAK3	3	MSH6	4	RB1	12	TSC1	1
CDKN2A	4	EZH2	1	GNAQ	1	KDR	6	NOTCH1	1	RET	6	VHL	3

56G panel genes and number of amplicons per gene. Hotspot loci (white), contiguous overlapping coverage (blue), and comprehensive coding exon coverage for TP53 (darker blue).

Two Liquid Biopsy Assays: Genome-Wide Hypomethylation and Mutation Detection

| mL Plasma	ng/mL cfDNA	Pathology	cfDNA Hypomethylation	56G Mutation	Normal Adjacent	FFPE Tumor	cfDNA
2.5	6.3	Fallopian tube high-grade papillary serous carcinoma	0.4%	TP53 E285K	0%	48%	0%
5.0	4.3	5 cm ovarian 'borderline' serous content	1.1%	BRAF V600E	0%	14%	0%
3.8	4.4	Recurrent pT2, pN0 mammary carcinoma	2.4%	PIK3CA H1047R	0%	17%	0%
4.0	10.5	pT1/pN1 pancreatic adenocarcinoma	3.6%	-	-	-	-
3.0	6.7	Metastatic colon cancer to the liver	4.4%	-	-	-	-
4.5	7.1	14 cm ovarian 'borderline' serous content	18.0%	BRAF V600E	0%	23%	1%
4.5	2.6	Colon-cancer, non-resectable adenocarcinoma	18.0%	TP53 frameshift exon 8	0%	15%	2%
4.5	2.9	Metastatic colorectal adenocarcinoma	43.4%	PIK3CA E545K	0%	23%	11%
				APC Q1429*	0%	20%	5%
				TP53 Q38*	0%	21%	14%
				KRAS G13D	0%	22%	5%

A correlation between cfDNA hypomethylation and detection of tumor mutations in cfDNA may exist. Significant hypomethylation was detected in 6 of the 8 samples, and the 56 gene amplicon panel identified point mutations in the cfDNA of the three samples with the highest observed hypomethylation. Concordance was observed between corresponding cfDNA and FFPE tumors, when mutations were detected.
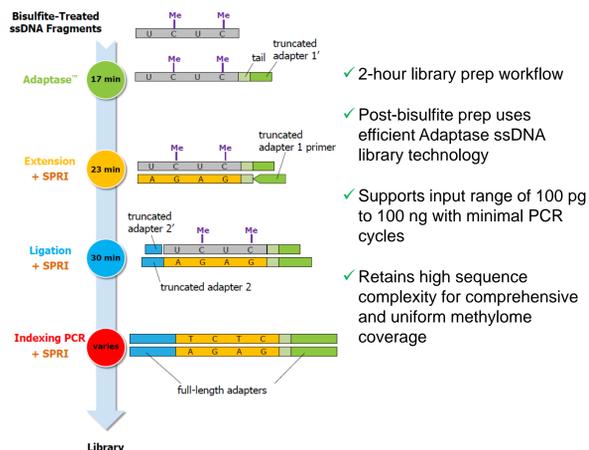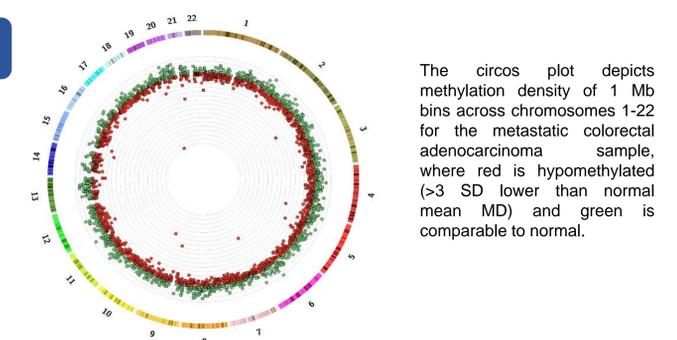
Limit of Detection (gDNA)

1 ng of gDNA = 334 chromosomal copies of any locus
Achieving 1% detection of a mutation → 3 chromosomal copies

Sample 1 — 0.50% / 99.50% — A, B
Sample 2 — 1% / 99% — A, B
Sample 3 — 0.50% / 99.50% — A, B
Sample 4 — 1% / 99% — A, B
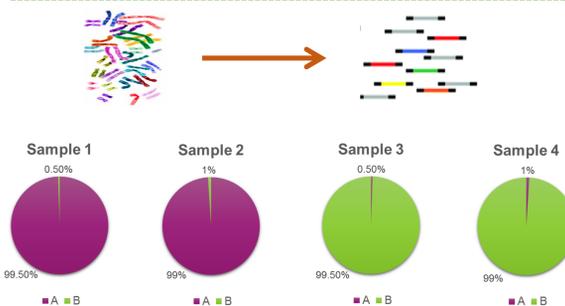
To assess the limit of detection of the Accel-NGS 2S Hyb Kit, DNA samples from two individuals (A and B) with different ethnic backgrounds were used to prepare libraries. 100 ng of DNA from one individual with a 0.5% or 1% spike-in of the DNA from the second individual was used as the input DNA. Once libraries were prepared, they were hybridized to xGen® Pan-Cancer probes and SNPs were detected within this panel.

cfDNA Input	Allele Frequencies				
CHR: POS	A Background	B Background	C Background	1% A into 10 ng B	1% A into 10 ng C
2: 212244718	100%	0%	0%	0.6%	1.0%
12: 25361074	100%	0%	0%	1.6%	1.9%
12: 25361142	100%	0%	0%	1.1%	0.9%
12: 25361646	100%	0%	0%	1.9%	1.6%
12: 40688695	100%	0%	0%	0.5%	1.1%
12: 115108136	100%	0%	0%	0.7%	2.0%

To determine if SNPs present at 1% allele frequency could be detected, 1% of cfDNA sample (A) with a unique ethnic background was spiked into two 10 ng cfDNA samples (B and C) of different ethnic backgrounds. SNPs at 100% from sample A could be detected around 1% when those SNPs are not present in B and C backgrounds. Libraries were sequenced to an average coverage of 8700X.

The circos plot depicts methylation density of 1 Mb bins across chromosomes 1-22 for the metastatic colorectal adenocarcinoma sample, where red is hypomethylated (>3 SD lower than normal mean MD) and green is comparable to normal.

Conclusions

Accel-NGS Methyl-Seq DNA Library Kit:
- Provides uniform, comprehensive methylome coverage.
- Enables liquid biopsy for genome-wide hypomethylation from 5 ng cfDNA.

Accel-Amplicon 56G targeted sequencing panel:
- Provides quality performance with > 95% on target and > 95% coverage uniformity.
- Enables a limit of mutation detection of 1% for liquid biopsy from 10 ng cfDNA.

Using both kits for liquid biopsy, a correlation was observed between percent hypomethylation and mutation detection for the tumor bearing cfDNA sample set presented.

The Accel-NGS 2S DNA Library Kit:
- Provides uniform, comprehensive genome coverage.
- Enables PCR-free cfDNA sequencing from 10-15 ng input.

Disclosure: All authors are employees of Swift Biosciences, Inc.

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