A multiplexed amplicon sequencing technology for FFPE and circulating, cell-free DNA

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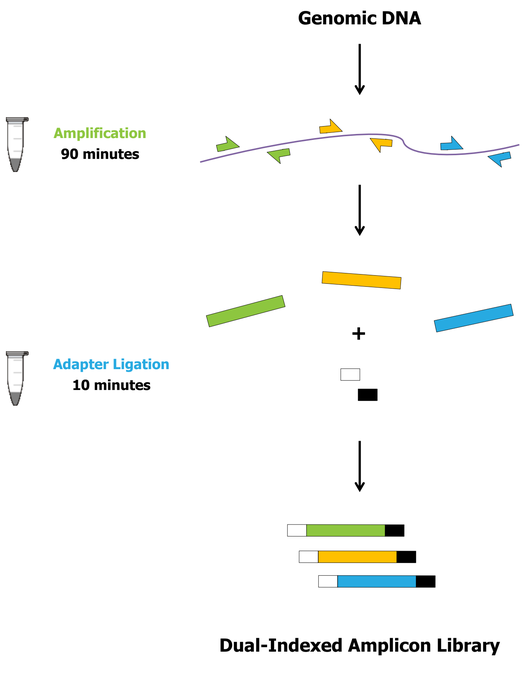
Accel-Amplicon™

Abstract

Detection of somatic mutations is challenging since percent tumor content in clinical samples is variable and is compounded by tumor heterogeneity. Additionally, circulating cfDNA and FFPE samples are typically limited in quantity, and FFPE samples can also be highly damaged. To address these challenges, we developed a single-tube, multiplexed amplicon sequencing method that employs hundreds of primer pairs for amplification of target loci, producing ready-to-run libraries for Illumina® sequencing. The two-step method- multiplexed PCR followed by a 10 minute adapter ligation- results in amplicons 120-160 bp in length, enabling amplification and variant calling from cfDNA-sized DNA fragments or damaged FFPE DNA. Using this technology, an oncology panel was developed to target known, clinically relevant mutations in 56 genes. For example, EGFR resistance was targeted to enable liquid biopsy monitoring in cfDNA samples. The panel design encompasses single exons (e.g. BRAF) as well as comprehensive coding exon coverage of entire genes (e.g. TP53), depending on the allele distribution across each target gene. To validate this panel, a cohort of control and clinical samples with pre-validated genotypes was tested using 10 ng of input DNA. Amplicon libraries were quantified by qPCR and sequenced on a MiSeq. Alignment and variant calling were performed using validated, publicly available tools and confirmed manually. Robust detection of 5% mutant frequency was observed for samples, and in performing spike-in experiments, the limit of detection was as low as 1% mutant frequency. The percent on-target bases and coverage uniformity were both >95%, where uniformity is defined as the percent bases covered at >20% of the mean coverage. These results indicate that this multiplexed amplicon panel is an excellent tool to assess multiple oncogenes in limiting clinical samples, enabling high throughput, cost effective NGS analysis.

Accel-Amplicon™ workflow



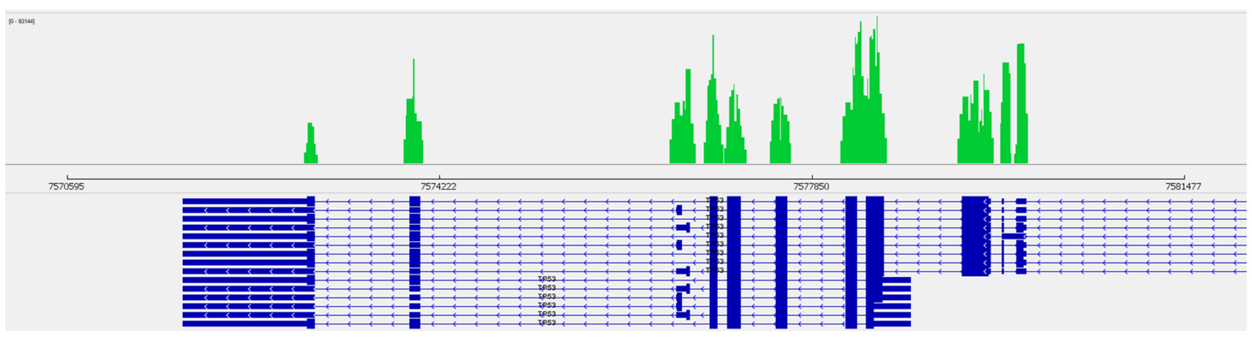
- Accel-Amplicon™ has 2 steps:
  1. Multiplexed PCR plus bead-based purification
  2. Adapter ligation plus bead-based purification
- Single tube amplification for hundreds of primer pairs, including overlapping amplicons
- Proprietary method eliminates primer dimer "noise" of multiplexed PCR
- High fidelity polymerase for low amplification error
- Amplicon size 120-160 bp to enable sensitivity for FFPE and cfDNA samples
- Illumina® sequencing
- 10 ng input DNA required
- 2 hour turnaround, start to finish
- Fast, consistent processing

Accel-Amplicon™ 56G Oncology Panel

ABL1	5	CSF1R	2	FBXW7	6	GNAS	2	KIT	14	NPM1	1	SKT11	5
AKT1	2	CTNNB1	1	FGFR1	2	HNF1A	4	KRAS	3	NRAS	3	SMAD4	10
ALK	2	DDR2	1	FGFR2	4	HRAS	2	MAP2K1	5	PDGFRA	4	SMARCB1	14
APC	9	DNMT3A	1	FGFR3	6	IDH1	1	MET	6	PIK3CA	11	SMO	5
ATM	19	EGFR	9	FLT3	4	IDH2	2	MLH1	1	PTEN	14	SRC	1
BRAF	2	ERBB2	4	FOXL2	1	JAK2	2	MPL	1	PTPN11	2	TP53	21
CDH1	3	ERBB4	8	GNA11	2	JAK3	3	MSH6	4	RB1	1	TSC1	1
CDKN2A	2	EZH2	1	GNAQ	2	KDR	9	NOTCH1	3	RET	6	VHL	3

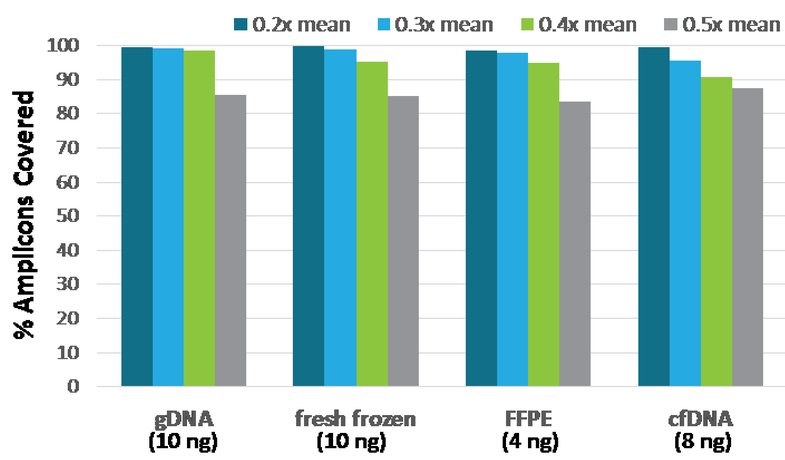
The 56G Oncology Panel includes both clinically relevant hotspot loci and regions of contiguous coverage, depending on the allele distribution across each target gene. The table depicts the genes represented, followed by the number of amplicons for each. Contiguous, overlapping coverage is included for APC, ATM, EGFR, FBXW7, FGFR3, HNF1A, KIT, MSH6, PIK3CA, PTEN, SMAD4 and TP53. Comprehensive coding exon coverage is included for TP53. The total targeted region is ~24 Kb.

Comprehensive coding exon coverage of TP53



The annotated TP53 isoforms from IGV are depicted in blue. Coding exon coverage by the 21 overlapping amplicons from the 56G Oncology Panel are depicted in green, where the highest depth of coverage is achieved at amplicon overlap.

High coverage uniformity across sample types



For Accel-Amplicon™ libraries, >95% reads were on target. At an average read depth of 2000X, coverage uniformity was achieved across all four sample types, regardless of sample quality and fragment size. Input DNAs were quantified by ALU repeat qPCR for accurate measure of amplifiable content. gDNA, fresh frozen and FFPE contained HMW DNA whereas the cfDNA contained a narrow 165 bp size distribution.

Validation of variant calling on AcroMetrix® Oncology control DNA

DNA input	% > 20% mean	expected variants	observed @ >5000X	observed @ 2000X
10 ng	97%	472	472	467
10 ng	97%	472	470	459
1 ng	97%	472	463	462
1 ng	97%	472	467	466

Validation of variant calling for the 56G Oncology Panel was performed using AcroMetrix® Oncology control DNA, where 10 ng or 1 ng total input DNA was used. The control DNA has 521 variants from the COSMIC database across 53 genes at either a 5-15% frequency or a 15-35% frequency. The 56G Oncology Panel covers 472 of these variants. The table depicts the expected and obtained results for these alleles at the recommended 10 ng input as well as 1 ng input DNA. Observed variants called was performed at both 2000X average coverage depth and at >5000X average coverage depth. Variant calling was performed using GATK and LoFreq (Wilm *et al*., NAR 2012).

Variant calling as low as 1% sensitivity

Gene	Normal	SW480	5% spike	1% spike	0.5% spike
KRAS G12V	**WT**	**99.8%**	**6.8%**	**0.7%**	**0.63%**
TP53	4.2%	-	4.2%	3.4%	4%
TP53 P309S	**WT**	**99.7%**	**1.9%**	**0.7%**	**3.1%**
TP53 R273H	**WT**	**99.8%**	**3.3%**	**1.1%**	**-**
TP53	1%	-	1.1%	1.4%	0.99%

Variant calling limit of detection for the 56G Oncology Panel was determined by sequencing normal human DNA with varied percent spike-in of mutant cell line SW480 DNA, where 10 ng total input DNA was used. All three SW480 mutants (bold text) could be detected at 5% and 1% spike-in but results were variable for 0.5%. Two low frequency SNPs observed in the normal DNA persisted across all replicates, demonstrating reproducibility of low variant calls using this technology. Variant calling was performed using LoFreq.

Comparison to AmpliSeq on FFPE tumor samples

Ion AmpliSeq™	Location TP53	Accel-Amplicon™
V225I	7577608	✓
R248Q	7577538	✓
L130F	7578542	✓
G244D	7577550	✓
R273H	7577120	✓
E286K	7577082	✓
M246I	7577543	✓
R306X	7577022	✓
Y107X	7579366	✓
E180K	7578392	✓
D148H	7578488	✓
R248W	7577539	✓
~~E204G~~	7578238	WT
~~Y205H~~	7578236	WT
~~Del~~	7579373	WT

Variant calling from clinical samples was determined for the 56G Oncology Panel and compared with an Ion AmpliSeq™ Panel on cervical tumor FFPE and matched normal blood reference samples. 10ng input DNA was used per sample and sequencing depth performed at 1000X average coverage. The AmpliSeq assay called mutations across TP53 coding exons in 15 tumor samples. Using GATK Unified Genotyper for variant calling, mutations were called using Accel-Amplicon in 12 tumor samples. The discrepant calls were subsequently determined to be AmpliSeq false positives (red strikethrough). These three samples were properly called as TP53 WT by Accel-Amplicon. The normal blood reference samples had no TP53 mutations except for the patient carrying TP53 V225I which was a germline mutation found in both tumor and normal (data not shown). Samples and AmpliSeq data were obtained from the National Cancer Institute (NCI).

Accel-Amplicon™ summary

- Multiplexed amplicon sequencing technology with a simple, single tube workflow
- 56G Oncology Panel covers clinically relevant hotspots and larger contiguous regions
- Exceptional uniformity of coverage from HMW gDNA, FFPE and cfDNA
- Variant calling validated using Acrometrix DNA standard and pre-validated clinical FFPE tumors
- Limit of detection as low as 1% mutant frequency from 10 ng
- Limit of detection as low as 5% mutant frequency from 1 ng

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