Accurate, High Sensitivity Next Generation Sequencing of Targeted Cancer Genes in FFPE and FNA Clinical Specimens

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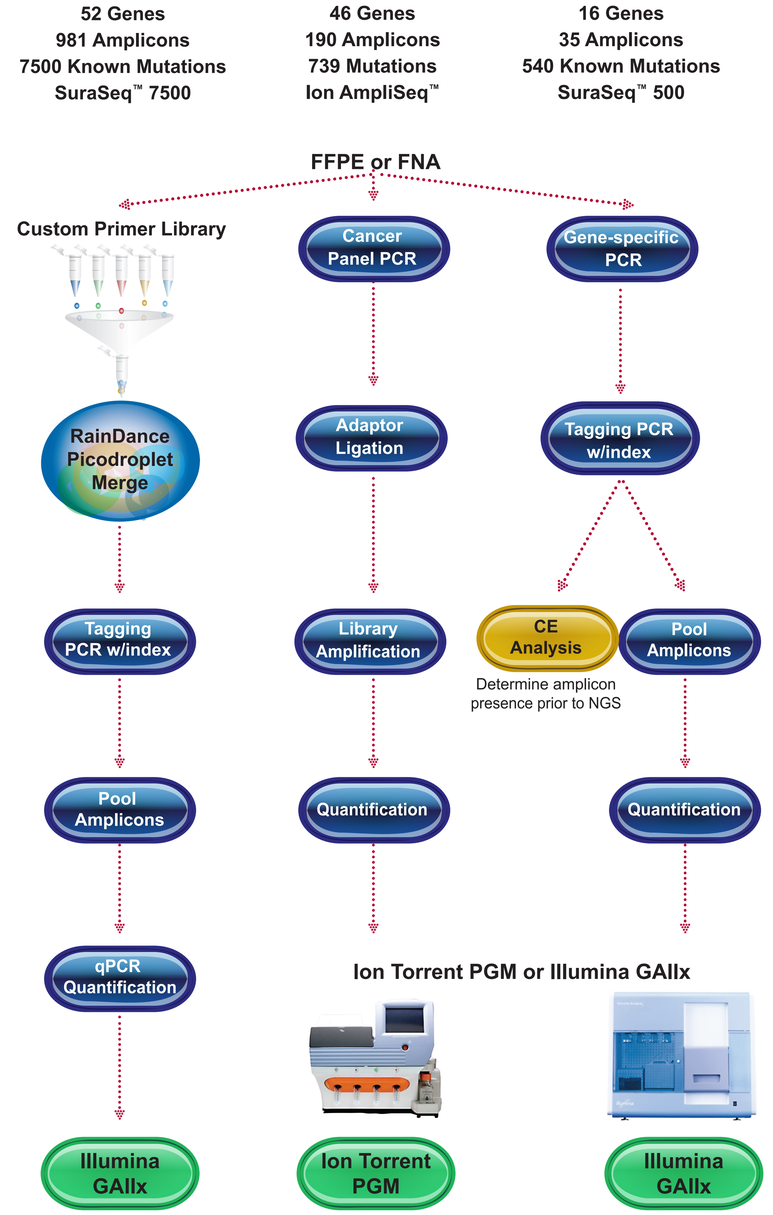
SUMMARY

- Procedures for direct amplicon sequencing on two orthogonal Next Generation Sequencing (NGS) platforms were developed to enrich for mutations from clinically actionable gene regions using FFPE cancer specimens.
- The various enrichment procedures enabled uniform coverage across both focused (35 amplicon), expanded (190 amplicon) and broad (981 amplicon) content panels, with NGS read depths of >1000X and detection of variants representing <5% of reads.
- The results support the utility of high sensitivity, high resolution mutation assessments across thousands of loci in heterogeneous FFPE tumor specimens.
- Samples tested by both the PGM and GAIIx platforms were also highly concordant across all sample types with orthogonal enrichment protocols

MATERIALS AND METHODS

Three FFPE-compatible PCR-based enrichment panels were developed and tested. The first was a multiplexed PCR assay that targeted 35 amplicons in 16 cancer genes, including the most common mutations in the MAPK/ERK and PI3K/AKT pathways. The second was an expanded 46 gene and 190 amplicon panel commercialized by Life Technologies (Ion AmpliSeq™). The third included nearly 1000 amplicons from 52 cancer genes enriched using the RainDance RDT-1000. Primers were designed to avoid known SNPs, repetitive sequences, and pseudogenes whenever possible, and included adaptor sequences to enable direct sequencing on either the Ion Torrent PGM or the Illumina GAIIx. FFPE DNA inputs from 10 ng to 2 ug were evaluated for PCR enrichment, and samples were barcoded to enable sample multiplexing of up to 36 samples/lane (GAIIx) or 13 samples/chip (PGM). Workflows for GAIIx NGS required ~3-6 weeks, whereas sample processing on the PGM required <3 days.



16 Cancer Gene Targeted NGS – SuraSeq™ 500

ABL1	FGFR1	HRAS	MET
AKT1	FGFR3	JAK2	NRAS
BRAF	FLT3	KIT	PDGFRA
EGFR	RET	KRAS	PIK3CA

Table 1. A 16 gene, 35 amplicon panel represents >95% of all mutations in these genes indexed in COSMIC and 540 known mutations.

DNA Sample	Gene with known mutations	Known Codon Change	Type	Mixing ratio	Expected % Mutation	Ion Torrent PGM % Recovered	Ion Torrent PGM Read Coverage	Illumina GAIIx % Recovered	Illumina GAIIx Read Coverage
A-549	KRAS	G12S	HOM	35%	**35**	**31.9**	971	**29.2**	4072
MIA PaCa-2	KRAS	G12C	HOM	20%	**20**	**22.6**	971	**17.8**	4072
T24	HRAS	G12V	HOM	10%	**10**	**10.9**	248	**13.2**	4424
RKO	BRAF	V600E	HET	15%	**7.5**	**5.9**	801	**6.5**	7772
	PIK3CA	H1047R			**10.5**	**6.6**	2290	**7.9**	5896
SK-Mel-2	NRAS	Q61R	HOM	7%	**7**	**10.7**	600	**11.2**	7150
GP2d	PIC3CA	H1047L	HET	5%	**2.5**	**3.5**	2290	**6.8**	5896
	KRAS	G12D			**2.5**	**3.7**	991	**4.0**	4072
HCT 116	KRAS	G13D	HET	6%	**3**	**1.5**	1024	**<0.5**	4072
	PIK3CA	H1047R			**10.5**	**6.6**	2290	**7.9**	5896
SW1116	KRAS	G12A	HET	2%	**1**	**1.4**	991	**1.3**	4072

Table 2. Ion Torrent PGM and Illumina GAIIx NGS quantitatively recovers known mutations from pooled cancer cell DNA.

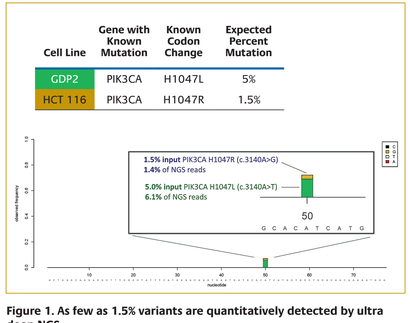


Figure 1. As few as 1.5% variants are quantitatively detected by ultra deep NGS.

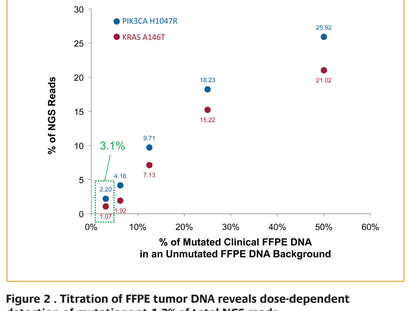


Figure 2 . Titration of FFPE tumor DNA reveals dose-dependent detection of mutations at 1-2% of total NGS reads.

Concordance Summary for FFPE Samples Across KRAS, BRAF, and PIK3CA Hotspots		Orthogonal Confirmation	
		POS	Wt
Illumina GAIIx	POS	19	3
	Wt	2	92
Ion Torrent PGM	POS	15	0
	Wt	1	91

Table 3. Illumina GAIIx NGS of 39 FFPE tumor specimens demonstrates 96% concordance and Ion Torrent PGM NGS of 16 FFPE tumor specimens demonstrates 99% concordance with confirmation assays, including Signature® assays run on the Luminex platform and Sanger Sequencing.

Concordance Summary for 20 FNA Samples Across RAS and BRAF Hotspots		Orthogonal Confirmation	
		POS	Wt
PGM	POS	13	1*
	Wt	1**	80

Table 4. PGM NGS of 20 FFPE FNA tumor specimens demonstrates 98% concordance with orthogonal confirmation assays.* Based on NGS read coverage, below LOD by Sanger Sequencing; ** detected with a probe-based assay with < 1% LOD. Re-biopsy of patient was negative.

Category	Intact DNA (N=27)	FFPE DNA (N-=39)
Average Depth	38,500	24,500
Maximum	72,738	44,747
Minimum	1,784	3,432
Range within 5-fold of average	95%	95%
Median Variant	**0.27%**	**0.3%**

Table 5. Both intact cell line DNA and FFPE DNA demonstrate A) uniform read coverage, and B) low level of background base substitution variants (0.3%) on Illumina GAIIx.

46 Cancer Gene Targeted NGS: Ion AmpliSeq™

KRAS	BRAF	EGFR	TP53	PIK3CA	CSF1R	JAK2
NRAS	PTPN11	ERBB2	SRC	FGFR3	NPM1	CDKN2A
RET	HNF1A	SMAD4	GNAS	PDGFRA	MPL	ABL1
PTEN	FLT3	STK11	SMARCB1	KIT	MET	NOTCH1
FGFR2	RB1	JAK3	VHL	KDR	SMO	
HRAS	AKT1	ALK	MLH1	FBXW7	ERBB4	
ATM	CDH1	IDH1	CTNNB1	APC	FGFR1	

Table 6. The Ion AmpliSeq™ panel assesses 739 known mutational hot spots across 46 genes.

Sample ID	Total Reads	Median Coverage	Percentage within 5x of median	Known Mutations	Detected by Ion AmpliSeq™
FFPE #1	347,328	1137	78%	BRAF V600E	BRAF V600E
FFPE#2	255,720	953	80%	KRAS A146T PIK3CA H1047R	KRAS A146T PIK3CA H1047R
FFPE#3	514,351	1894	81%	NRAS Q61K	NRAS Q61K
FFPE#4	382,035	1329	81%	KRASG12D	KRASG12D
FFPE#5	307,626	891	75%	None	None

Table 7. Ion AmpliSeq™ Cancer Panel Enrichment followed by Ion Torrent NGS detects cancer mutations in residual FFPE biopsies consistent with independent methods. Barcoding was not available for these sample runs.

Mutations	Expected % Mutation	Ion AmpliSeq™ 316 Op1		Ion AmpliSeq™ 314 Op2	
		% Recovered	# of Reads	% Recovered	# of Reads
KRASG12C	20	18.6	14945	20.6	1124
TP53 R248W	20	15.1	6816	14.1	1033
HRAS G12V	10	15.9	1132	15.7	159
TP53 Y126	10	12.2+	4784+	7.7+	988+
BRAF V600E	7.5	9.4	22294	6.9	1212
NRAS Q61R	7	12.4	8178	11.6	1417
TP53 G245S	3.5	4.3+	7398+	3.1+	1024+
KRAS G12D	2.5	5.6	15295	5.2	1135
KRAS G13D	3	1.9	18602	1.2	1490
KRAS G12A	1	1.8	15295	2.2	1472
TP53 A159D	2	4.4+	2108+	1.8+	334+
KRAS G12S	35	34.1	14945	32.2	1124
STK11 Q37*	35	31.2	9269	29.2	995

+Recovered using NextGENE analysis

Table 8. Ion AmpliSeq™ Cancer Panel Enrichment followed by Ion Torrent NGS quantitatively recovers known mutations from pooled cancer cell DNA. R^2=0.98 for comparison of quantitative mutation detection between Op1 on 316 chip and Op2 on 314 chip. Barcoding was not available for these sample runs.

52 Cancer Gene Targeted NGS – SuraSeq™ 7500

ABL1	DNMT3A	GNAQ	MET	**PTCH1**	**TP53**
AKT1	**EGFR**	**HIF1A**	MPL	**PTEN**	**VHL**
AKT2	ERBB2	HRAS	**NF2**	PTPN11	
BRAF	FES	IDH1	**NOTCH1**	**RB1**	
CDH1	FGFR1	IDH2	NPM1	RET	
CDK4	FGFR3	IKBKB	NRAS	**SMAD4**	
CDKN2A	FLT3	JAK2	PAX5	**SMARCB1**	
CEBPA	FOXL2	**KIT**	PDGFRA	**SMO**	
CREBBP	GATA1	**KRAS**	PIK3CA	SRC	
CTNNB1	GNA11	**MEN1**	**PIK3R1**	**STK11**	

Table 9. A 52 gene, 981 amplicon panel represents 7500 known mutations indexed in cosmic. Genes shown in bold text were sequenced across all exons.

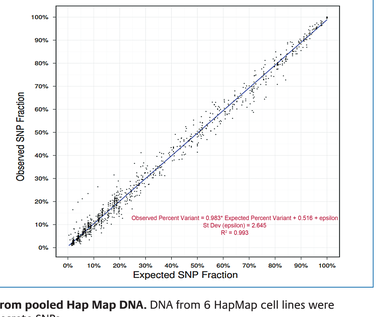


Figure 3. A 981 amplicon panel recovers the full range of expected SNP fractions from pooled Hap Map DNA. DNA from 6 HapMap cell lines were combined at 6 different mass mixing ratios to produce 1254 data points across 209 discrete SNPs.

Sample	HapMap1	2	3	4	5	6
CM0040	0.31	**0.27**	**0.16**	0.14	**0.08**	**0.04**
CM0041	0.03	0.38	0.24	**0.17**	0.11	0.07
CM0042	0.07	0.04	0.36	**0.26**	**0.16**	0.12
CM0043	**0.1**	0.08	0.04	0.4	0.23	0.16
CM0044	0.14	0.13	0.09	0.05	**0.36**	0.24
CM0045	**0.21**	0.18	0.12	0.09	**0.04**	0.36

		PGM		GAIIx		
		SuraSeq™ 500	Ion AmpliSeq™	SuraSeq™ 500	SuraSeq™ 7500 (100 ng)	SuraSeq™ 7500 (1000 ng)
PGM	SuraSeq™ 500	1	0.90	0.96	0.80	0.94
	Ion AmpliSeq™	0.90	1	0.96	0.93	0.95
GAIIx	SuraSeq™ 500	0.96	0.96	1	0.83	0.97
	SuraSeq™ 7500 (100 ng)	0.80	0.93	0.83	1	0.90
	SuraSeq™ 7500 (1000 ng)	0.94	0.95	0.97	0.90	1
Known Input		0.97	0.93	0.91	0.88	0.89

Table 10. R^2 correlation matrix of orthogonal enrichment methods. Correlations were derived from variant quantification using pooled cancer cell line DNA representing known mutations (Tables 2,8).

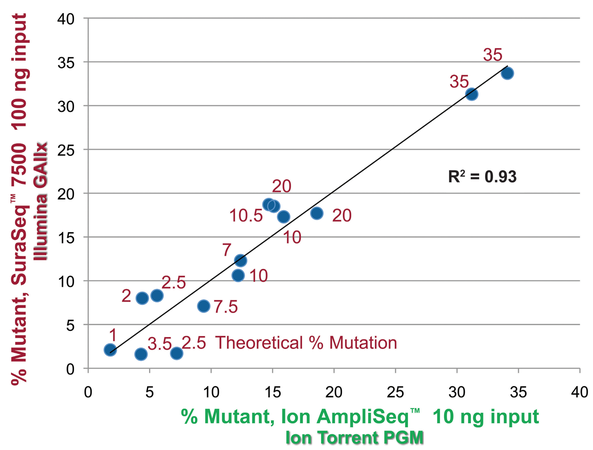


Figure 4. Percent mutation detected by 100 ng DNA into SuraSeq™ 7500 on GAIIx (Y) vs 10 ng DNA input on Ion AmpliSeq™ Ion Torrent (X).

CONCLUSIONS

- Three distinct PCR workflows enabled high depth enrichment of cancer-associated gene regions in FFPE and FNA DNA from clinical specimens.
- Mutation loads as low as 1-3% could be accurately identified in both cancer cell line and FFPE tumor DNA; “background” variant detection was only ~0.3%.
- Ion Torrent NGS successfully confirmed novel mutations from screening studies using the Illumina GAIIx, suggesting utility for high sensitivity orthogonal mutation confirmation using a second NGS system.
- SuraSeq™ cancer gene panels supported a streamlined protocol, low DNA inputs, multiplex target amplification, and, importantly, efficient multi-sample barcoding, even on the Ion Torrent PGM.
- The three proposed NGS approaches can accommodate both large-scale, whole exon mutation assessments in ~96 samples per run, as well as “hotspot” mutation analyses across 15-50 genes with a rapid turnaround time (<1 week).