Two Complementary and Scalable PCR-based Workflows Enable Next Generation Sequencing of Cancer-Associated Genes in FFPE Tumor DNA

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SUMMARY

- Next generation sequencing (NGS) has the potential to report low abundance, clinically actionable mutations in heterogeneous, real-world tumor specimens.
- Multiplex PCR enrichment methods were developed with both focused (16 genes, 35 amplicon) and broad (52 genes, 981 amplicon) cancer gene panels for direct sequencing on two orthogonal NGS platforms, the Illumina GAIIx and the Ion Torrent Personal Genome Machine (PGM).
- The two enrichment methods enabled uniform read coverage, high depth sequencing (>1000X), high sensitivity mutation detection (1-3%), and excellent concordance with Sanger sequencing and other mutation confirmation methods using FFPE samples from different tumor types and block ages.
- The results support the utility of sensitive, accurate, and high resolution mutation profiling across dozens to thousands of loci in FFPE tumor specimens.

MATERIALS AND METHODS

Two FFPE-compatible PCR-based enrichment panels were developed. 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 included nearly 1000 amplicons from 52 cancer genes. Primers were designed to avoid known SNPs, repetitive sequences, and psuedogenes whenever possible, and included adaptor sequences to enable direct sequencing on either the Ion Torrent PGM or the Illumina GAIIx. Samples included cancer cell lines (ATCC) and residual FFPE blocks from various sources collected up to 14 years prior and represented colon, melanoma, breast, and other cancers. FFPE DNA inputs ranging from 10 ng to 2 ug were evaluated for PCR enrichment, and samples were barcoded up to 36plex/lane (GAIIx) or 13plex/chip (PGM). Workflows for GAIIx NGS required ~3-6 weeks, whereas the time from purified DNA to processed NGS data on the PGM was <3 days.

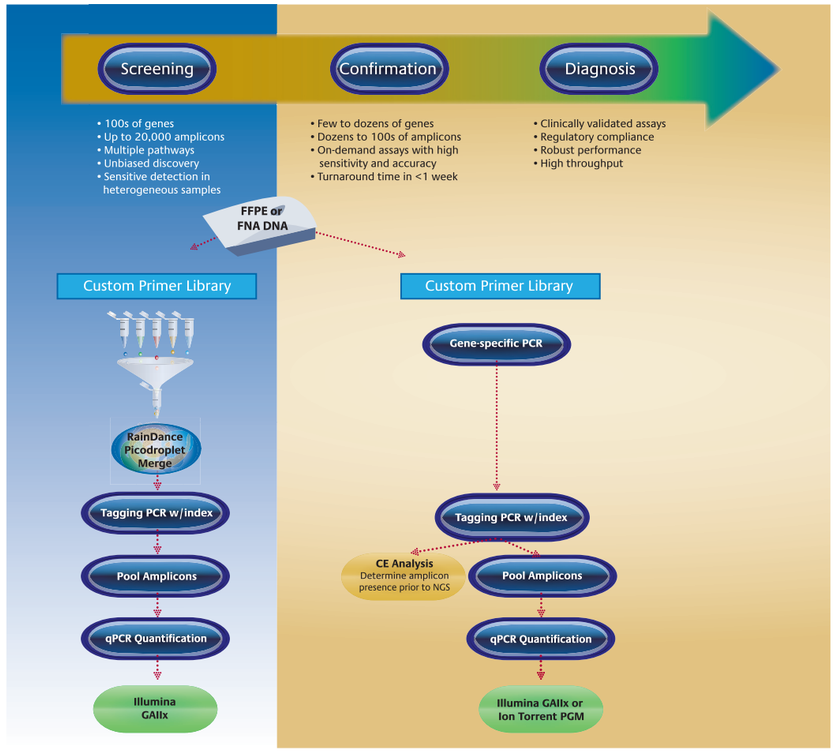


Figure 1. Two distinct PCR enrichment workflows with NGS applications ranging from broad content screening to molecular diagnostics.

16 Gene Cancer Panel

Oncogene Panel			
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 these gene mutations in COSMIC.

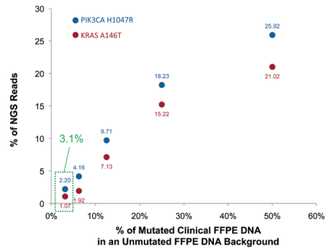


Figure 3. Titration of FFPE tumor DNA reveals dose-dependent detection of mutations to 1-2% of total NGS reads.

Concordance Summary for 39 FFPE Samples across KRAS, BRAF, and PIK3CA Hotspots		Sanger Sequencing	
		POS	Wt
Illumina GAIIx	POS	19	3
	Wt	2	92

Table 3. Illumina GAIIx NGS of 39 FFPE tumor specimens demonstrates 96% concordance with Sanger sequencing.

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

Concordance Summary for 16 FFPE Samples across RAS, BRAF, and PIK3CA Hotspots		Orthogonal Confirmation	
		POS	Wt
Ion Torrent PGM	POS	15	0
	Wt	1	91

Table 5. Ion Torrent PGM NGS of 16 FFPE tumor specimens demonstrates 99% concordance with confirmation assays, including Luminex Signature®, Illumina and Sanger sequencing.

Cell Line	Gene with Known Mutation	Known Codon Change	Expected Percent Mutation	NGS Percent Mutation
GDP2	PIK3CA	H1047L	5%	6.1%
HCT116	PIK3CA	H1047R	1.5%	1.4%

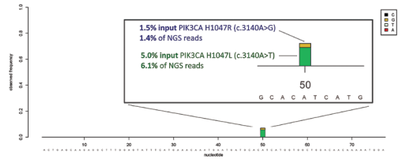
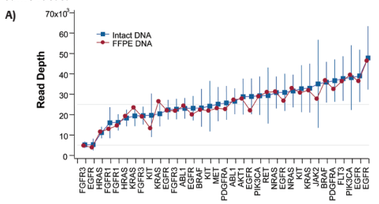


Figure 2. As few as 1.5% variants can be accurately quantified by ultra deep NGS using the Illumina GAIIx: co-detection of PIK3CA variants at the same locus.



B)

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%**	**0.32%**

Table 2. Both intact cell line DNA and FFPE DNA demonstrate A) uniform read coverage, and represent B) low levels of "background" base substitution variants (0.3%).

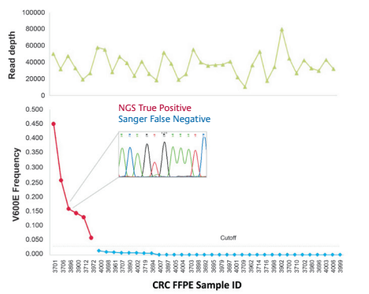


Figure 4. High depth NGS of BRAF amplicons clearly delineates mutation positive samples, including those that are negative by Sanger sequencing.

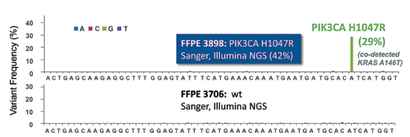


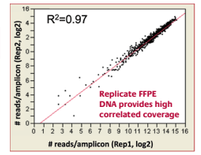
Figure 5. Representative variant profile of mutation-positive and negative FFPE tumor DNA revealed by Ion Torrent NGS. Mutations identified on the Illumina GAIIx were confirmed on the Ion Torrent PGM.

52 Gene Cancer Panel

Oncogene and Tumor Suppressor Panel					
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**	

Figure 6. A 52 gene, 981 amplicon cancer panel compatible with massively parallel picodroplet PCR. Amplicons designed for genes shown in bold were sequenced across all coding exons.

Metric	CRC FFPE1	CRC FFPE1 (replicate)
Total Reads	7,654,272	5,601,478
Median reads/amplicon	6375	4471
2-fold of median	57%	57%
5-fold of median	91%	91%



Metric	CRC FFPE2 (1 ug)	CRC FFPE2 (2 ug)
Total Reads	3,180,016	7,835,776
Median reads/amplicon	2682	6438
2-fold of median	65%	62%
5-fold of median	96%	95%

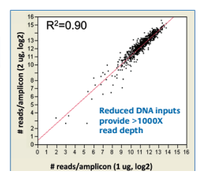


Figure 7. Picodroplet PCR of FFPE DNA using a 981 amplicon cancer panel supports reproducible, ultra deep NGS using the Illumina GAIIx.

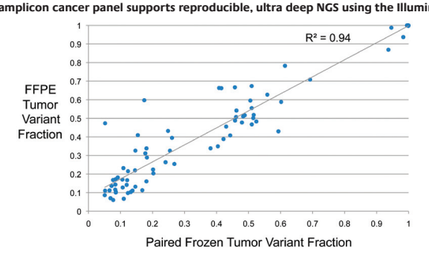


Figure 8. Variant fractions detected by NGS in matched frozen and FFPE tumor DNA are highly correlated. Variants were identified by Illumina GAIIx NGS following RainDance enrichment of 981 amplicons spanning 52 cancer genes.

CONCLUSIONS

- Two distinct PCR workflows enabled high depth and enabled uniform enrichment and high depth NGS of cancer-associated gene regions in FFPE DNA from residual clinical specimens.
- Mutation loads as low as 1-3% were accurately identified in both cancer cell line and FFPE tumor DNA; "background" variant detection was only 0.3%. These results have important implications for detection of low-level mutations, such as drug-resistant mutations, that may be clinically relevant.
- PCR-based enrichment of cancer gene "hotspots" in FFPE tumors revealed 96-99% concordance with orthogonal reference methods, including Sanger sequencing.
- Ion Torrent NGS successfully confirmed novel mutations from screening studies using the Illumina GAIIx, suggesting utility for high sensitivity orthogonal mutation confirmation using distinct enrichment procedures and NGS sequencing chemistries.
- The two proposed NGS approaches can accommodate both large-scale, whole exon mutation assessments for ~96 samples per run, as well as "hotspot" mutation analyses across more than a dozen genes with a rapid turnaround time (<1 week).