# High Sensitivity Detection of Clinically Actionable Mutations in FFPE and FNA Tumor Biopsies Using Two Orthogonal Next Generation Sequencing Procedures

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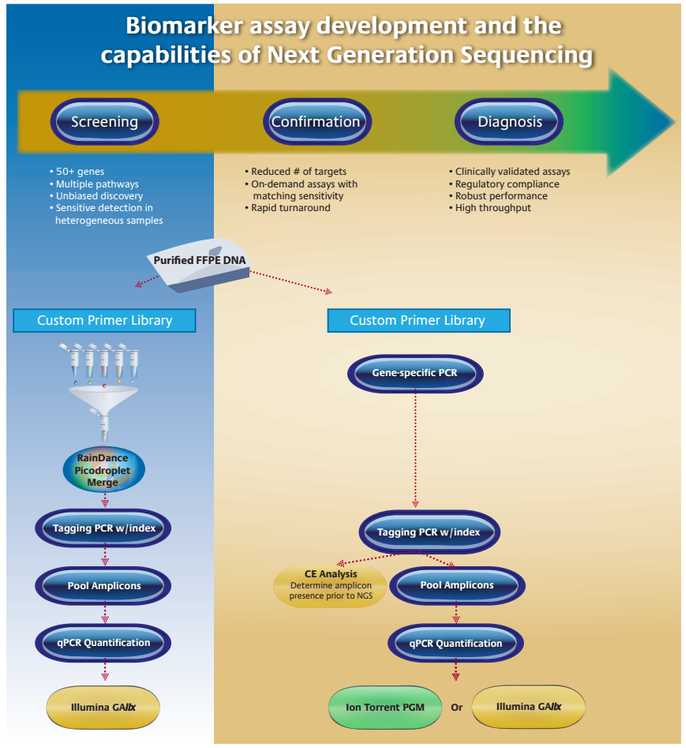
## SUMMARY

- Next generation sequencing (NGS) can provide comprehensive and massively parallel analysis of molecular alterations in oncogenes and tumor suppressor genes associated with cancer pathway signaling.
- Complementary and scalable enrichment procedures for direct amplicon sequencing of "druggable" cancer genes were developed for FFPE and Fine Needle Aspiration (FNA) specimens across two orthogonal NGS platforms.
- PCR enrichment procedures enabled uniform coverage across SuraSeq™ 200, SuraSeq™ 500, SuraSeq™ 7500 and Ion AmpliSeq™ cancer gene panels, with NGS read depths of >1000X and detection of variants representing as few as 1-3% of reads.
- The results support the utility of high sensitivity, high resolution mutation assessments across thousands of loci in heterogeneous FFPE and FNA tumor specimens.

## MATERIALS AND METHODS

Three FFPE and FNA compatible PCR-based enrichment panels were developed. The first two were multiplexed PCR assays that targeted 8 amplicons in 5 cancer genes or 35 amplicons in 16 cancer genes, including the most common mutations in the MAPK/ERK and PI3K/AKT pathways. The third included nearly 1000 amplicons from 52 cancer genes. 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 GA/*Ix*. FNA DNA inputs from 10 ng or FFPE DNA inputs from 10 ng to 2 ug were evaluated for PCR enrichment, and samples were barcoded up to 36/lane (GA/*Ix*) or 13/chip (PGM). Workflows for GA/*Ix* NGS required ~3-6 weeks, whereas sample processing on the PGM required <3 days.




## SuraSeq™ 200

| | |
|---|---|
| BRAF | NRAS |
| HRAS | PIK3CA |
| KRAS | |

**Table 1. A 5 gene, 8 amplicon panel represents 211 unique mutations indexed in the COSMIC database.**

| Concordance Summary for 10 FFPE Samples across RAS, BRAF, and PIK3CA Hotspots | | Orthogonal Confirmation | |
|---|---|---|---|
| | | POS | Wt |
| PGM | POS | 9 | 0 |
| | Wt | 0 | 71 |

**Figure 1. Ion Torrent PGM of 10 FFPE specimens demonstrates 100% concordance with orthogonal assays.**

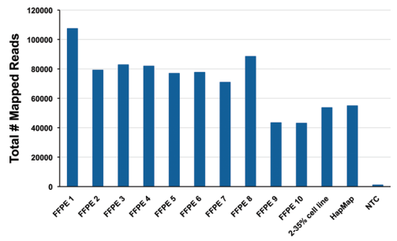



**Figure 2. Total mapped reads on a 316 chip across 13 barcoded specimens using the Ion Torrent PGM.** NTC = Non template control.

## SuraSeq™ 500

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

**Table 2. A 16 gene, 35 amplicon panel represents 540 unique mutations and >95% of all mutations in these genes listed in COSMIC.**

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

*Below LOD by Sanger sequencing.
**Only detected with a probe-based assay with < 1% LOD; Re-biopsy was negative.

**Figure 3. Ion Torrent PGM NGS of 20 FNA specimens demonstrates 98% concordance with confirmation assays, including Sanger sequencing.**

| 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 4. Both intact cell line DNA and FFPE DNA demonstrate low levels of "background" base substitution variants (0.3%).**

| 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 3. NGS of Ion Torrent and Illumina GA/*Ix* qualitatively and quantitatively recovers known mutations from pooled cancer cell DNA.**

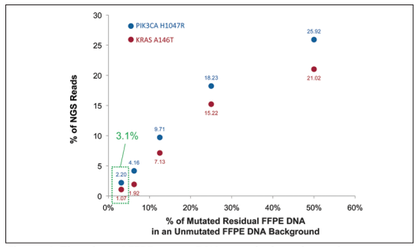



**Figure 4. Titration of FFPE tumor DNA reveals dose-dependent detection of mutations at 1-2% of total NGS reads.**

## Ion AmpliSeq™

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

**Table 4. Ion Torrent AmpliSeq™ is a 46 gene, 190 amplicon panel that represents 739 mutations.**

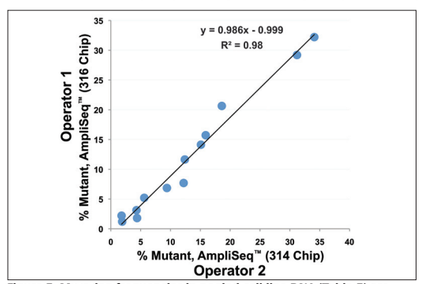



**Figure 5. Mutation frequencies in pooled cell line DNA (Table 5) are highly correlated across operators and sequencing chips.** AmpliSeq™ multi-sample barcoding was not available for the samples processed.

| DNA Sample | Gene with Known Mutations | Known Codon Change | Type | Mixing Ratio | Expected % Mutation | Ion Torrent AmpliSeq™ % Recovered | Ion Torrent AmpliSeq™ Read Cov (316) |
|---|---|---|---|---|---|---|---|
| A-549 | KRAS | G12S | HOM | 35% | **35** | **34.1** | 14945 |
| | SKT11 | Q37* | | | | **31.2** | 9269 |
| MIA PaCa-2 | KRAS | G12C | HOM | 20% | **20** | **18.6** | 14945 |
| | TP53 | R248W | | | | **15.1** | 6816 |
| T24 | HRAS | G12V | HOM | 10% | **10** | **15.9** | 1132 |
| RKO | BRAF | V600E | HET | 15% | **7.5** | **9.4** | 22294 |
| | PIK3CA | H1047R | | | **10.5** | **14.7** | 566 |
| SK-Mel-2 | NRAS | G61R | HOM | 7% | **7** | 12.4 | 8178 |
| GP2d | PIK3CA | H1047L | HET | 5% | **2.5** | **7.2** | 566 |
| | KRAS | G12D | | | **2.5** | **5.6** | 15295 |
| HCT 116 | KRAS | G13D | HET | 6% | **3** | **1.9** | 18602 |
| | PIK3CA | H1047R | | | **10.5** | **14.7** | 566 |
| SW1116 | KRAS | G12A | HET | 2% | **1** | **1.8** | 15295 |

**Table 5. Ion AmpliSeq™ enrichment quantitatively recovers known mutations from pooled cancer cell DNA following PGM 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** | |

**Figure 6. A 52 gene, 981 amplicon panel represents 7500 unique mutations. Amplicons designed for genes shown in bold were sequenced across all coding exons.**

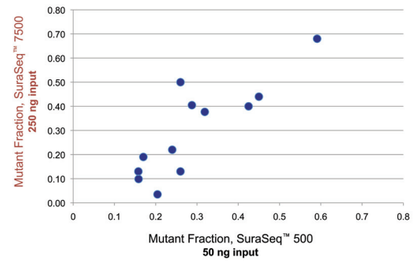



**Figure 8. Twelve known cancer gene mutations from 12 residual clinical FFPE samples are highly correlated after enrichment with SuraSeq™ 7500 and SuraSeq™ 500 panels and NGS on different sequencing systems.**

| 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 |

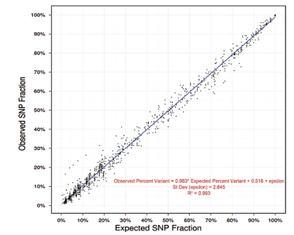



**Figure 7. SuraSeq™ 7500 recovers the full range of expected SNP fractions from pooled HapMap DNA in a Latin square titration, identifying 209 SNPs (for each of six cell lines) across 1254 data points.** Mixing ratios were designed such that the majority of SNPs were <20% abundance. Expected SNP fraction is based on results from individually sequencing each HapMap cell line.

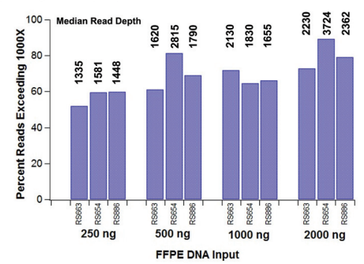



**Figure 9. As little as 250 ng FFPE DNA supports high depth NGS following SuraSeq™ 7500 enrichment.**

| | | PGM | | GA/*Ix* | | | |
|---|---|---|---|---|---|---|---|
| | | SuraSeq™ 200 | SuraSeq™ 500 | Ion AmpliSeq™ | SuraSeq™ 500 | SuraSeq™ 7500 (100ng) | SuraSeq™ 7500 (1000ng) |
| PGM | SuraSeq™ 200 | 1 | 0.96 | 0.94 | 0.98 | 0.81 | 0.97 |
| | SuraSeq™ 500 | 0.96 | 1 | 0.90 | 0.96 | 0.80 | 0.94 |
| | Ion AmpliSeq™ | 0.94 | 0.90 | 1 | 0.96 | 0.93 | 0.95 |
| GA/*Ix* | SuraSeq™ 500 | 0.98 | 0.96 | 0.96 | 1 | 0.83 | 0.97 |
| | SuraSeq™ 7500 (100ng) | 0.81 | 0.80 | 0.93 | 0.83 | 1 | 0.90 |
| | SuraSeq™ 7500 (1000ng) | 0.97 | 0.94 | 0.95 | 0.97 | 0.90 | 1 |
| Known Input | | 0.93 | 0.97 | 0.93 | 0.91 | 0.88 | 0.89 |

**Figure 10. Mutation quantification using distinct PCR enrichment panels and NGS platforms is highly correlated using known input cancer gene mutations for pooled cell line DNA (Tables 3, 5).**

## CONCLUSIONS

- Three distinct PCR workflows enabled high depth enrichment of cancer-associated gene regions in FFPE and FNA DNA from residual clinical specimens.
- Mutation loads as low as 1-3% can be accurately identified in both cancer cell line and FFPE tumor DNA; "background" variant detection was only ~0.3%.
- PCR-based enrichment of cancer gene "hotspots" in FFPE and FNA tumors revealed concordance in mutation detection using orthogonal methods.
- 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. Thus, SuraSeq™ focused gene panels offered high depth sequencing for multiple samples per run, compared to lower depth reads for singleplex AmpliSeq™.
- The three proposed enrichment 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).