Obtaining Maximal Information from Limited FFPE Tissue Specimens: Development and Validation of a 4-Gene Custom NGS Panel for a GIST Clinical Trial

Peng Fang, Zhenyu Yan, Jessica Kristof, Justin Staha, Kim Pelak, Michael R. Palmer*, Catherine Tribouley*, Cindy Spittle, Chad Galderisi, and Jin Li

MolecularMD Corp., Cambridge, MA and Portland, OR; *Novartis, Cambridge, MA

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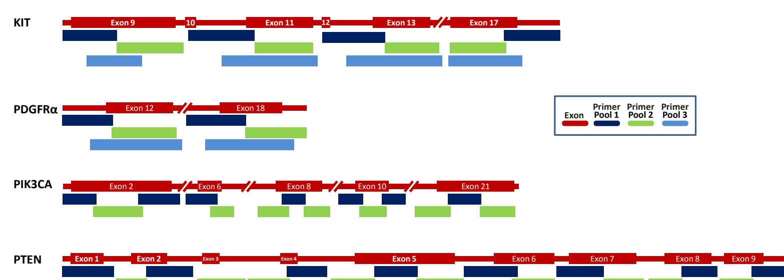
Introduction

Limited tissue availability and FFPE sample quality present challenges for cancer mutation profiling, particularly for solid tumors. Maximizing molecular information from specimens requires advanced profiling techniques; focused NGS panels provide higher sensitivity and more comprehensive sequence coverage than other methods and enable multiplexed examination of several genes related to a given therapy.

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm of the gastrointestinal tract. The incidence of GIST is estimated to be approximately 10-20 per million individuals per year worldwide (3000–4500 cases/year in the US). Mutations in c-KIT or PDGFRα are the hallmarks of GIST diagnosis. An estimated 80-85% of GIST cases contain mutations in c-KIT, and 5-10% of GIST cases contain mutations in PDGFRα. The type and the location of the mutations in c-KIT and PDGFRα have been shown to be associated with varied drug responses in GIST patients. The association of the mutations in other genes such as PIK3CA and PTEN with drug responses in GIST patients remains to be established.

A custom GIST panel was designed on the Ion Torrent platform to profile c-KIT, PDGFRα, PIK3CA and PTEN, using minimal DNA from FFPE tissue, which may aid in the interpretation of drug responses.

Figure 1. ROI targeted by the Custom GIST panel – Schematic of Amplicons by Primer Pool



Materials and Methods

The 2.1 kb regions of interest (ROI) include exons 9, 11, 13, 17 of c-KIT, exons 12 and 18 of PDGFRα, exons 1-9 of PTEN, and exons 2, 6, 8, 10, 21 of PIK3CA, as well as the intronic 3 bp at intron-exon boundaries (**Figure 1**, **Table 1**). Three pools of the primers to sequence the ROI were designed primarily by Ion AmpliSeq Designer, supplemented with MolecularMD's proprietary primer design method to ensure 100% coverage of the ROI and all of the known critical hotspots for GIST. HapMap sample NA12878 was used as the Negative Control. A mixture of DNA from NA12878 and two human cancer cell lines was used as the Positive Control, in which three mutations were expected to be detected: one single base substitution (SBS) mutation in KIT, one SBS mutation in PIK3CA, and one 1 bp deletion in PTEN.

Three libraries were prepared for each DNA sample (10 ng DNA input per library) with Ion AmpliSeq Library Kit, using three separate pools of primers. Each library was uniquely barcoded. The libraries were amplified on the OneTouch system using OneTouch OT2 200 Kit, enriched on the Ion One Touch ES, and sequenced on the Ion PGM using the Ion PGM Sequencing 200 Kit v2 and a 316 chip. The sequencing was performed in batches, with no more than 32 libraries and two chips per batch. A positive control and a negative control were included in each batch. The sequencing data were analyzed with Torrent Suite 3.4.2 and MolecularMD's proprietary analysis pipeline.

A series of experiments was conducted to validate the following five parameters: 1) the primer specificity was assessed through bioinformatics analysis; 2) the assay specificity was assessed by the analysis of 6 FFPE normal (non-tumor) tissue samples; 3) the assay limit of detection (LOD) of SBS and indels was determined by using serial dilutions of the Positive Control DNA into the Negative Control DNA; 4) the assay reproducibility was evaluated through inter-run assay replicate analysis with cell-line DNA and 3 FFPE DNA samples; 5) the accuracy of the assay was examined by comparison of the results of the NGS assay to results from Sanger sequencing with 30 FFPE tumor DNA samples. **Table 2** summarizes the FFPE specimens used.

Table 1. ROI targeted by the Custom GIST panel

Genes	Exons	Amino Acid Residue Covered
KIT	9	449-514
KIT	11	550-592
KIT	13	627-664
KIT	17	788-828
PDGFRα	12	552-596
PDGFRα	18	814-854
PIK3CA	2	1-118
PIK3CA	6	354-382
PIK3CA	8	418-468
PIK3CA	10	514-555
PIK3CA	21	979-1069
PTEN	1-9	1-404

Table 2. The FFPE samples used in the Validation Study

Study	Tissue	Type	Number
Specificity study	Colon	Normal	4
	Appendix	Normal	1
	Jejunum	Normal	1
Precision study	Colon	Normal	1
	Colon	Tumor	1
	GIST	Tumor	1
Accuracy study	Uterus	Tumor	9
	Stomach	Tumor	8
	Esophagus	Tumor	1
	GIST	Tumor	12

Results

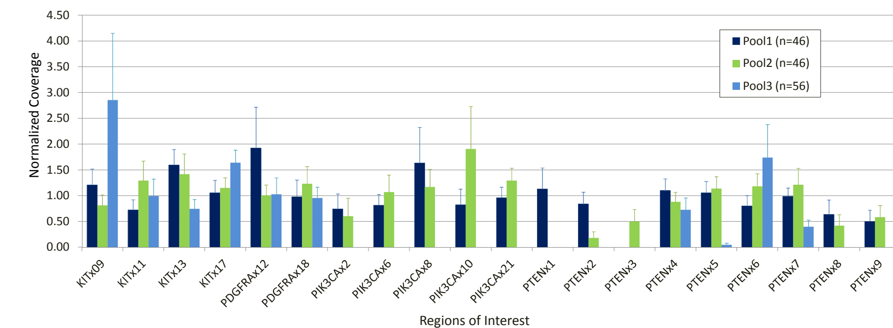
ROI and Library Coverage

The mean coverage of all the libraries prepared during the validation study was ≥ 1287 (**Table 3**). Libraries prepared from cell line DNA had higher coverage than libraries prepared from the FFPE DNA, due to the higher integrity of the cell line DNA. 148 qualified libraries were prepared from FFPE DNA samples and analyzed. 51/53 amplicons were sequenced with greater than or equal to 0.2x normalized coverage (**Figure 2**). The PTENx2 region that is sequenced by pool 2 may have sufficient raw coverage to call variants, depending on the overall coverage of the library. The PTENx5 region that is sequenced by pool 3 has sufficient overlapping coverage by pools 1 and 2.

Table 3. Coverage of all libraries prepared

Type of DNA	No. of Libraries	Coverage (X)		
		Lowest	Highest	Median
FFPE	148	1287	89340	8212
Cell Line	64	4125	33603	9641

Figure 2. Normalized ROI Coverage in the libraries prepared from FFPE DNA



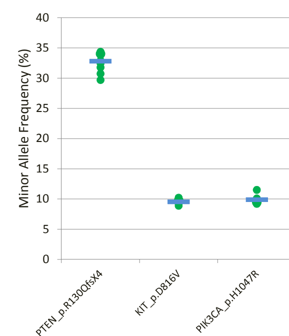
QC metrics

In all of the sequencing runs carried out in the validation study, all three of the expected mutations in the Positive Control (**Table 4**) were reproducibly detected (**Figure 3**). None of these runs had mutations called in the Negative Control.

Table 4. Expected mutation frequencies in Positive Control

Gene Name	cDNA Change	Amino Acid Change	Expected Allele Frequency
PTEN	c.389delG	p.R130QfsX4	30%
KIT	c.2447A>T	p.D816V	10%
PIK3CA	c.3140A>G	p.H1047R	10%

Figure 3. Range of allele frequencies of the mutations detected in the Positive Control (n=10)



Assay LOD

A series of dilutions of mixed DNA from cell lines harboring KIT and PIK3CA SBS mutations and a PTEN 1bp deletion was prepared to produce a set of samples with decreasing mutation frequencies. All of the mutations expected in serial dilutions were reproducibly detected in three sequencing runs by three operators (**Table 5**). Both SBS and the 1bp deletion were detected at ≤5% in all 3 runs.

Table 5. LOD study results

Gene Name	cDNA change	Amino Acid Change	Sequencing Results	Dilution A	Dilution B	Dilution C	Dilution D
PTEN	c.389delG	p.R130QfsX4	Expected Frequency (%)	30	15	7.5	3.75
			Observed Frequency (%)	32.3 ± 2.3	18.7 ± 1.6	9.6 ± 1.2	4.9 ± 0.6
			Coverage (x)	6745 ± 1859	5615 ± 1429	5750 ± 1970	5401 ± 1825
KIT	c.2447A>T	p.D816V	Expected Frequency (%)	10	5	2.5	1.25
			Observed Frequency (%)	9.7 ± 0.5	4.5 ± 0.5	2.3 ± 0.3	1.2 ± 0.1
			Coverage (x)	7817 ± 2682	7217 ± 2648	6933 ± 2829	6881 ± 2485
PIK3CA	c.3140A>G	p.H1047R	Expected Frequency (%)	10	5	2.5	1.25
			Observed Frequency (%)	9.6 ± 0.4	5.0 ± 0.6	3.0 ± 0.6	1.8 ± 0.3
			Coverage (x)	7817 ± 2682	7217 ± 2648	6933 ± 2829	6038 ± 1888

Assay Specificity

Six FFPE normal tissue samples were tested by a single operator using one 316 chip (data not shown). These FFPE samples were from normal tissue blocks, not from normal tissue dissected from tumor. No critical variants above the LOD of 5% were identified in the 6 FFPE normal tissue samples, validating the assay specificity.

Assay Accuracy

A total of 47 critical variants were detected in 28/30 FFPE samples. Two of the samples (2/30) did not have any critical variants. Among these 47 mutations, there were 28 SBS, 11 deletions, 3 deletions with insertions, and 5 insertions (**Figure 5**). The maximum observed deletion size was 51 bp, and the maximum observed insertion size was 45 bp. Variants were observed in all four of the targeted genes (**Figure 6**). All of the variants (46/46) from samples with sufficient DNA were confirmed by Sanger sequencing (false positive rate = 0%; representative traces in **Figure 7**). One variant could not be confirmed due to insufficient DNA for Sanger sequencing analysis.

Figure 5. Number of mutations detected by type

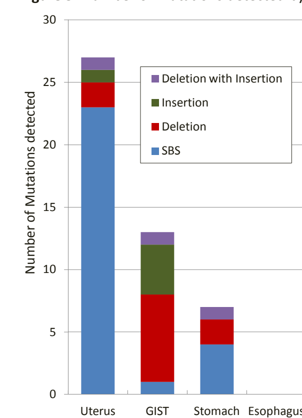
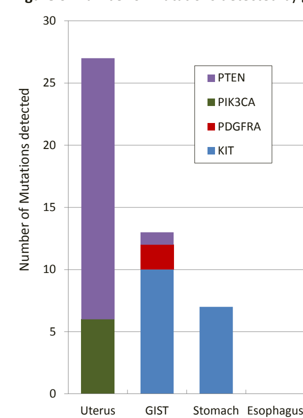


Figure 6. Number of mutations detected by gene



Assay reproducibility

A set of 8 samples including the Negative Control, the Positive Control, 1 wild-type FFPE sample, 2 FFPE mutant samples and 3 serial cell line dilutions were analysed on three runs, performed on three different days by distinct operators. All three mutations in the Positive Control and the three serial dilutions of the Positive Control were reproducibly detected in three sequencing runs by three operators (**Figure 4**). No critical variants were detected in any of the sequencing runs of the Negative Control or wt FFPE sample. All mutations, including the 2 SBS in one FFPE tumor DNA ("Sample 2" in **Figure 4**) and a 6 bp-deletion in another FFPE tumor DNA ("Sample 3" in **Figure 4**), were reproducibly detected in all three runs. For mutations with minor allele frequencies above 5%, the CV was <10%, and for mutations with minor allele frequencies below 5%, the CV was between 10% and 20%.

Figure 4. Precision Study with Positive Control and FFPE samples

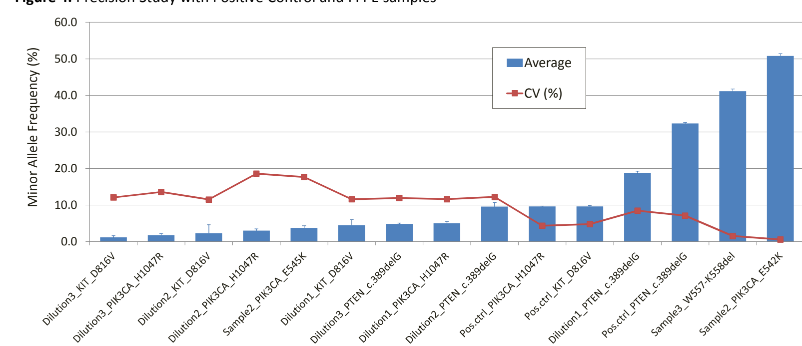
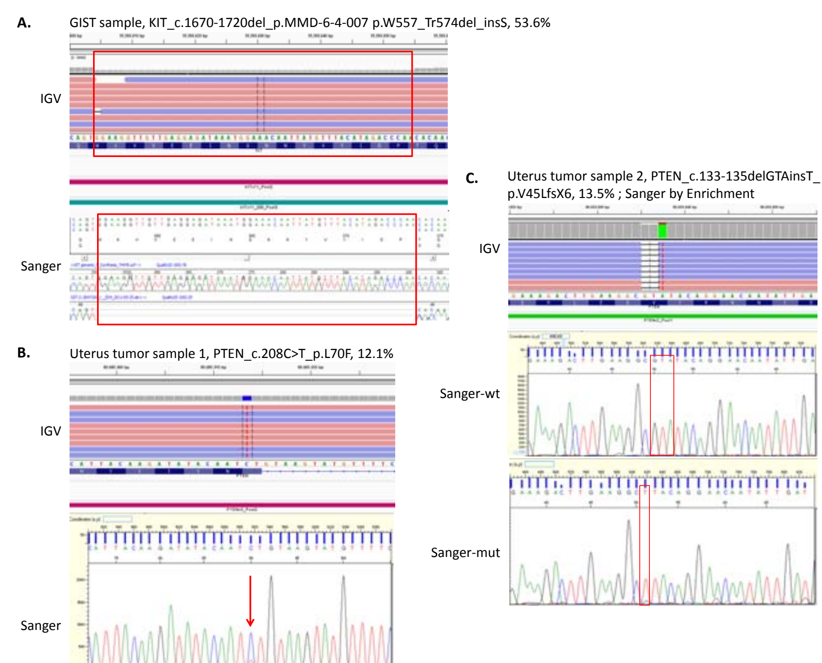


Figure 7. Examples of Sanger sequencing confirmation for (A) 51 bp deletion variant detected in KIT; (B) SBS variant detected in PTEN; (C) deletion with insertion (delGTAinsT) variant detected in PTEN



Conclusions

- This validation study demonstrates that with only 30 ng DNA input, the KIT-PDGFRα-PIK3CA-PTEN custom NGS Panel is capable of accurately detecting mutations, including SBS and small indels, in 20 exons across 4 genes, with a LOD of 5%, and false positive rate less than 5%.
- This custom panel is tailored to the demands of a targeted drug trial, and was designed to identify mutations in genes and regions most relevant to the interpretation of trial results.
- A custom NGS panel is scalable, in terms of ROI, size, and number of samples to be tested, and can be employed to maximize the breadth and depth of profiling information generated from a given tissue specimen.

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