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Small Customizable NGS Based Target Capture Panels Detect Variants in Clinical Specimens at Frequencies as Low as 0.5%

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Abstract

Background: The use of large scale hybridization panels in early stages of clinical trials for novel therapies elicits a plethora of information for targeted biomarkers. However, as therapeutic targets are further characterized large panels generate an overly broad set of data, compromising sensitivity in the selected biomarker subset. Therefore, once biomarker targets are identified, the use of smaller hybridization panels can facilitate specific variant detection by analyzing specific genomic regions of interest with greater sensitivity than larger gene panels and greater breadth than that available via PCR-based assays. Modifications of laboratory methods for small scale panels allow for the maintenance of high analytic quality with finely targeted panels. Our small panels (~10 kb) focus on 1-4 genes, allowing for high-multiplexing of samples on sequencers, and reduced costs/processing times without compromising accuracy.

Aims: To assess the sensitivity, linearity, and agreement of small NGS target capture panels with other assays.

Methods: Two separate next generation sequencing-target capture assays were developed with bioinformatics software. One panel contained 3 genes, including fms related tyrosine kinase 3 (*FLT3*); the second covers only *CD274* (*PD-L1*). Libraries were made, hybridized with baits, and sequenced. Testing was carried out by spiking in fixed amounts of mutant DNA into wild type DNA to determine the linearity and sensitivity of the assays. Sequencing libraries were generated by capturing with baits from either one or both panels. Sequencing data was analyzed using proprietary software developed by Invivoscribe. Eight AML clinical samples were tested for *FLT3* mutations by this small panel, amplicon based NGS assay, and capillary electrophoresis (CE) assay.

Results: DNA from 24 cell lines was assessed using both panels, confirming variants previously detected using other methods. A validation was run on the 3-gene panel using a series of contrived samples generated from cell lines containing between 0.5% and 25% variant allele frequencies for expected variants. Initial validation indicates that these small panel assays can detect mutations down to 0.5% variant allele frequencies. Assay linearity for *FLT3*/TKD detection from 0.25% to 12.5% or for *FLT3*/ITD detection from 0.5% to 25% is excellent (R^2= 0.996 and 0.998, respectively). Average sequencing coverage was high, ranging from 5,265x to 7,680x. Comparison of *FLT3* analysis of the small panel to amplicon based NGS assay and CE, *FLT3*-ITD showed a strong linear relationship between calculated VAFs and detected ITD sizes.

Conclusions: Small hybridization panels are cost effective in detecting low-frequency variants from smaller subsets of genes while using far less DNA than individual PCR-based biomarker assays would require. Preliminary data shows great accuracy on clinical samples. These smaller assays focus on the most pertinent genes for a targeted therapy, and have the potential to greatly assist in understanding the molecular backgrounds of responders, super-responders, and non-responders.

Materials and Methods

Library Preparation, Hybridization, Capture, and Washing: Whole-genome libraries were prepared and hybridized to probe sets.

Contrived Samples: 5 cell lines were used to generate contrived mixes of DNA with 4 expected variants against background DNA. Contrived mixes containing 25%, 10%, 5%, 2%, 1%, and 0.5% variant DNA were diluted in background DNA (DNA:DNA). Prior to dilution, input cell lines had expected mutations with variant allele frequencies (VAF) of 1 (Insertion 1), 0.5 (SNV 2), 0.5 (SNV 3), and 0.5 (SNV 4).

LoD, LoB, and Linearity: 3 replicates of 25% and 10% DNA:DNA dilutions, 4 replicates of 5%, 2%, 1%, and 0.5% DNA:DNA dilutions, and 2 replicates of background DNA were sequenced.

Precision and Reproducibility: 24 replicates of 5%, 2%, 1%, and 0.5% DNA:DNA dilution contrived samples were run through the 3-gene assay by 2 operators on 2 different days on 2 different instruments.

Comparison of Small Scale Hybridization Panel Results to Capillary Electrophoresis and *FLT3*-ITD-MRD Results: DNA from 8 subjects that had been previously analyzed by other methods were assayed using our small-panel hybridization assay. All 8 DNA samples had previously been analyzed using our capillary electrophoresis (CE) *FLT3*-ITD assay and *FLT3*-ITD-MRD assay. Assay results were compared for agreement.

Results: LOD, LOB and Linearity

To assess the LoD and linearity of our small target-capture panel assays, contrived samples were assayed using a target-capture panel covering 3 genes and 4 expected variants.

Linearity data is graphed in **Figure 1**. Each expected variant is graphed separately; and displays data from DNA:DNA% with 3 replicates for 25% and 10% and 4 replicates for 5%, 2%, 1%, and 0.5%. Equations for line of fit and R^2 values are listed on each graph. R^2 values are significant, ranging from 0.994 to 0.998.

LoD was established at 0.5% (**Table 1**). Of particular interest, we note that Expected SNV 4 is reliably at detected at a lower VAF of 0.25% (due to variable representation in the contrived sample).

No expected mutations were detected in 100% background DNA samples (N=2), LoB was established for each expected variant as the background variant rate + 5*stdev.

Figure 1: Linearity for 4 selected variants

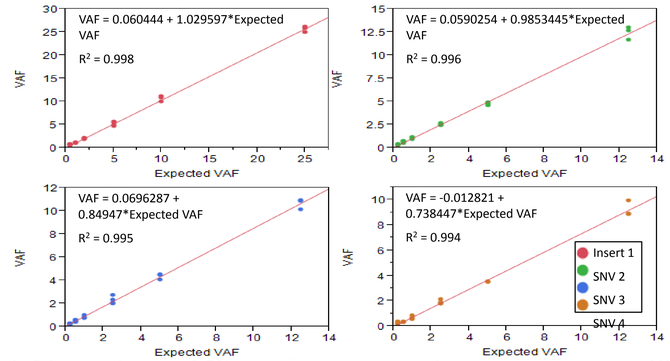


Table 1: LoD is established at 0.5%, and all expected mutations are observed at that VAF.

N	Expected Insert 1			Expected SNV 2			Expected SNV 3			Expected SNV 4		
	Expected VAF	VAF Range (%)	Tests positive for mutation (%)	Expected VAF	VAF Range (%)	Tests positive for mutation (%)	Expected VAF	VAF Range (%)	Tests positive for mutation (%)	Expected VAF	VAF Range (%)	Tests positive for mutation (%)
3	25%	25-26.3	100%	12.5%	11.6-13	100%	12.5%	10.1-10.9	100%	12.5%	8.8-10	100%
3	10%	10-11.1	100%	5.0%	4.6-4.9	100%	5.0%	4.1-4.5	100%	5.0%	3.47-3.52	100%
28	5%	4.7-5.6	100%	2.5%	2.4-2.6	100%	2.5%	2-2.7	100%	2.5%	1.8-2.1	100%
28	2%	1.8-2.2	100%	1.0%	1.1-1.2	100%	1.0%	0.7-1	100%	1.0%	0.6-0.9	100%
28	1%	0.9-1.2	100%	0.5%	0.5-0.7	100%	0.5%	0.4-0.6	100%	0.5%	0.3-0.4	100%
28	0.5%	0.5-0.8	100%	0.25%	0.3-0.4	67.9%	**0.25%**	**0.2-0.3**	**100%**	0.25%	0.2-0.4	64%

Results: Precision and Reproducibility

Libraries were generated by 2 operators on 2 days and were run on 2 different instruments. In total, 24 replicates each of 5%, 2%, 1%, and 0.5% (DNA:DNA) contrived samples were run.

For reproducibility, the acceptance criterion was set so no more than two 1% contrived samples were undetected. All contrived samples ≥1% DNA:DNA test positive for all 4 expected mutations, demonstrating excellent reproducibility of this assay.

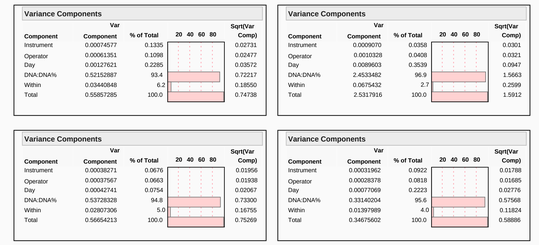
Precision analysis observed %CV of variant allele frequency (VAF), with the goal of this metric to be ≤30%. Data is presented in **Table 2**, and all expected variants pass this criterion.

Day-to-day, operator-to-operator, and instrument-to-instrument variation analysis is presented in **Figure 2**. Overall there is very little variance, most of which is from variations in DNA:DNA% and random (residual) sources.

Table 2: %CV of VAFs for all expected variants are below the cutoff of 30% for precision validation.

Contrived Sample (DNA:DNA%)	Expected VAF (%)	% CV of detected VAF (≤30%)			
		Expected Insertion 1	Expected SNV 2	Expected SNV 4	Expected SNV 3
5%	5	10.18			
	2.5		13.52	10.19	12.44
2%	2	8.36			
	1		12.31	13.55	9.56
1%	1	21.97			
	0.5		18.35	16.90	22.13
0.5%	0.5	28.48			
	0.25		16.28	20.46	21.93

Figure 2: Variance analysis of P/R data indicates very little variance stemming from random sources.



Results: Small Target Capture Panel is Well Correlated With Other Assays for *FLT3*-ITD

FLT3-ITD results from our small panel target capture panel were compared to other proprietary assays, our capillary electrophoresis, and our *FLT3*-ITD-MRD assay. We found 100% agreement among panels for 8 clinical samples tested for the *FLT3*-ITD insertion (Table 3). Additionally, the VAF and ITD size (bp) measured by both the CE assay and the MRD assay are highly correlated (Figures 3 and 4, respectively).

Table 3: ITD calls are consistent among the 3 assays tested

Sample	CE ITD	Small Panel - ITD	NGS-ITD-MRD - ITD
AML-03	-	-	-
AML-04	+	+	+
AML-05	+	+	+
AML-08	-	-	-
AML-09	+	+	+
AML-12	+	+	+
AML-18	+	+	+
AML-58	-	-	-

Figure 3: VAF is highly correlated among assays

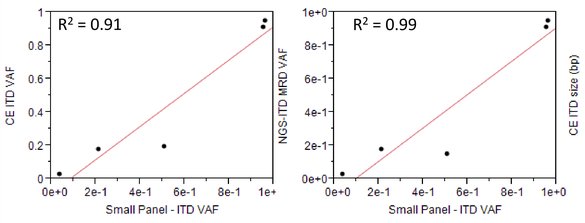
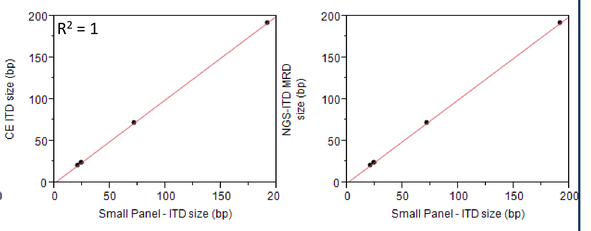


Figure 4: Insert size is highly correlated among assays



Conclusions

Small hybridization panels are cost effective in detecting low-frequency variants from smaller subsets of genes while using far less DNA than individual PCR-based biomarker assays. Small hybridization assays focus on the most pertinent genes for a targeted therapy and have the potential to greatly assist in understanding the molecular backgrounds of therapeutic monitoring. These panel based assays have high sensitivity and give highly comparable results to other NGS and CE based assays.

Data was generated for Research Purposes only.