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H02

Small Customizable Next-Generation Sequencing Based Target-Capture Panels in a Clinical Environment Can Detect Variant Mutations at Frequencies as Low as 0.5%

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Introduction

In clinical trials, large scale target-capture panels provide critical information for the selection of target biomarkers. However, as biomarker targets are further characterized large target-capture panels can elicit a glut of excess information, complicating analyses. Alternatively, once biomarker targets have been identified, the use of smaller, focused next-generation sequencing (NGS) based target-capture assays facilitate specific variant detection by sequencing genomic regions of interest with greater breadth than classic PCR-based assays. Moreover, laboratory methods for larger target-capture panels require extensive modification and optimization when applied to smaller scale target-capture panels to maintain optimal analytic quality. Here, we utilize smaller target-capture panels (~10kb) focusing on a few genes, allowing for high-multiplexing of samples on NGS platforms reducing cost per sample and decreasing processing time.

Here, we demonstrate excellent linearity, a Limit of Detection (LoD) of 0.5%, a location specific Limit of Blank (LoB), and good precision and reproducibility from small target-capture assays.

Materials and Methods

Library Preparation: Whole-genome libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems®).

Library Hybridization, Capture, and Washing: Whole-genome libraries were hybridized to probe sets (Integrated DNA Technologies®, Coralville, IA, USA) at 65°C overnight. Buffers from the SeqCap EZ Hybridization and Wash Kit (Roche®, Pleasanton, CA) were used for hybridization and washing.

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.

Results: LoD, LoB, and Linearity

To establish 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

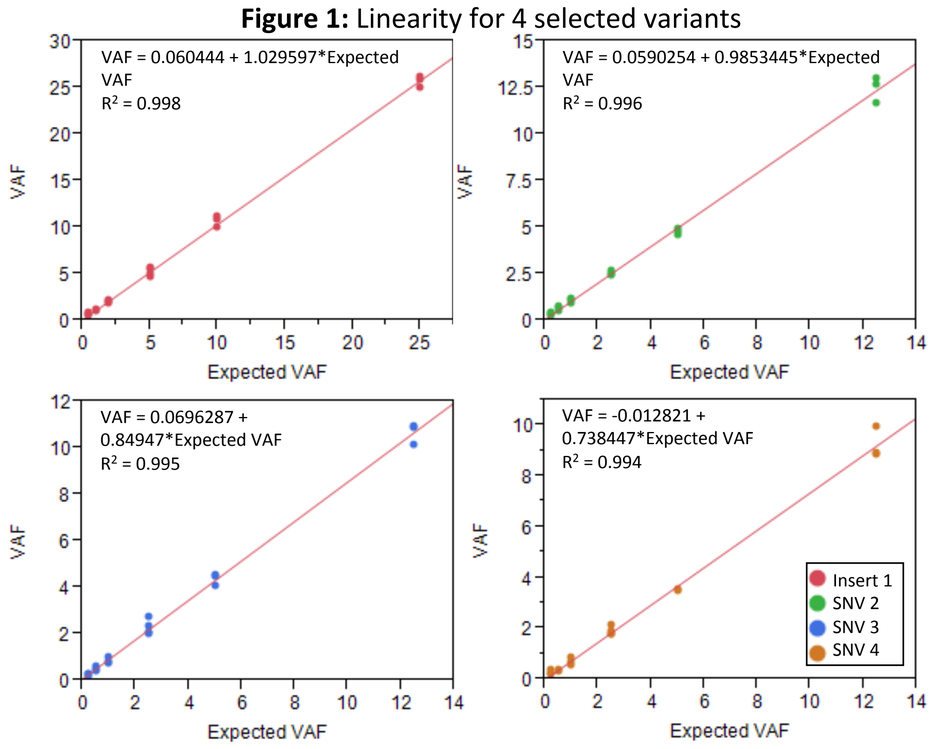


Table 1: LoD is established at 0.5%, and all expected mutations are observed at that VAF.

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.

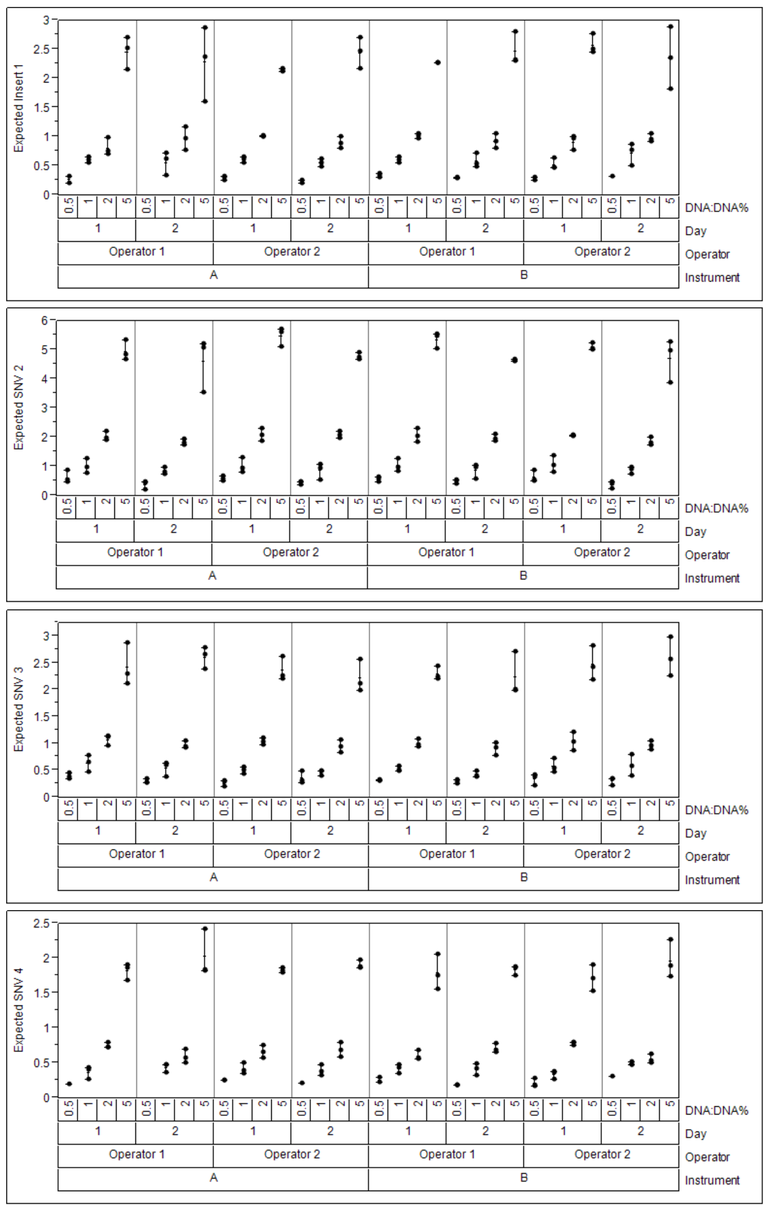
Precision analysis observed %CV (CV/$\bar{x}$) of variant allele frequency (VAF), with the goal of this metric to be ≤30%. Data is presented in **Table 2**, and all expected variants pass this criterion.

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.



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 responders, super-responders, and non-responders, information that can help improve patient outcomes. Developing NGS target-capture panels with bioinformatics in compliance with ISO 13485 and QSR design control requirements makes these assays suitable for pre-market submissions to worldwide regulatory authorities.

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