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Multiple Highly Concordant Assays Facilitate Analyses of Clinical Samples at Different Scales and Sensitivities

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Introduction

The uniform detection of clinically relevant mutations is critical to drug and diagnostics development. Technologies such as next-generation sequencing (NGS) have improved sensitivity of detection, but to date there is no "catch-all" method that addresses both the broad discovery phase of development and the more focused approach of target validation. We have developed several methods of variant detection to address this need for varied sensitivity and scale, utilizing technologies ranging from capillary electrophoresis (CE) to next-generation sequencing. For example, Invivoscribe's proprietary NGS large panel-based assays (such as MyHeme® and MyAML®) and NGS medium panel-based assays (MyMRD®) can be useful in early investigational stages of drugs and treatments, while highly targeted amplicon-based assays (*FLT3*-ITD MRD) can more specifically hone in on clinically relevant biomarkers in later stages of development. Here we demonstrate the ability to reliably detect clinically actionable *NPM1, FLT3*-ITD, and *FLT3*-TKD variants at varying frequencies in a set of clinical samples across multiple Invivoscribe-developed assays including CE single gene analysis, NGS-amplicon assays, and NGS small- to medium-scale targeted multi-variant panels.

Materials & Methods

Eight clinical AML samples from peripheral blood and bone marrow were tested at various dilutions using multiple Invivoscribe-developed CE, NGS-amplicon, and NGS-panel assays. Several dilutions of these samples were tested to demonstrate sensitivity of the NGS assays. An additional 30+ AML samples were run on the CE and NGS-amplicon assays for *FLT3* variants. In this study, small-scale NGS panels are defined as a few genes, and medium-scaled panels are 20+ genes. The DNA input for the assays ranged from 50-700 ng of high-quality genomic DNA. In NGS-Amplicon assays, regions containing variants were amplified by PCR. Up to 24 samples were purified, pooled, and sequenced. Target capture libraries were generated for NGS panel assays and sequenced. The Illumina MiSeq® platform was used for all NGS assays in this study. Sequencing data was analyzed using proprietary Invivoscribe MyInformatics® software, which produced variant read frequencies (VRF) defined as variant reads divided by total reads.

Table Legend	
CE	Capillary Electrophoresis
SR	Signal Ratio
VRF	Variant Read Frequency
+	Positive detection
-	Not detected
(grey)	Sample not tested

Results: Small Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS assays to identify a four base pair insertion in *NPM1*. Of the eight AML samples, five were positive for this insertion. The CE- based assay detected mutations in 2 out of 4 samples tested (Table 1), while the NGS assay identified *NPM1* insertions in five samples.

Table 1. Variant: *NPM1*

Sample Name	Dilution	CE Data	NGS-Amplicon		Medium-Scale NGS Panel	
		Call	Variant	VRF(%)	Variant	VRF(%)
AML-03	1	-	-	N/A	-	N/A
AML-04	1	+	insTGTA	46.9	insTGTA	37.6
	1/10		insTGTA	3.5	insTGTA	2.4
	1/100				insTGTA	0.2
AML-05	1		insTCTG	40.6	insTCTG	28.9
	1/10				insTCTG	4.3
AML-08	1		-	N/A	-	N/A
AML-09	1		insTCTG	46.1	insTCTG	34.7
	1/10		insTCTG	2.6	insTCTG	2.6
	1/100		insTCTG	0.3	insTCTG	0.3
AML-12	1	+	insTGCA	31.0	insTGCA	31.2
	1/10				insTCTG	3.9
	1/100				insTCTG	0.4
AML-18	1		insTCTG	34.6	insTCTG	38.2
	1/10				insTCTG	4.3
	1/100				insTCTG	0.4
AML-58	1	-	-	N/A	-	N/A

Results: Large Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS methodologies to identify internal tandem duplications (ITDs) in *FLT3*. Of the eight clinical samples examined, five were positive for the *FLT3*-ITD; all three assays detected *FLT3*-ITD at similar frequencies (Table 2). Following dilution of the clinical samples, low frequency mutations are no longer observable in the less sensitive CE assays. However, using the more sensitive NGS assays, variants are still detected at frequencies of 10^{-2} and 10^{-3}.

Table 2. Variant: *FLT3*-ITD

Sample Name	Dilution	CE Data				NGS -Amplicon		NGS-Panel Small-Scale		NGS-Panel Medium-Scale	
		Call	Size	SR	Var (%)	Size	VRF (%)	Size	VRF (%)	Size	VRF (%)
AML-03	1	-	N/A	N/A	N/A	-	N/A	-	N/A	-	N/A
AML-04	1	+	192.52	0.24	19	192	15	192	51	192	52
						51	0.05				
						24	0.02				
	1/10					192	0.4	192	4	192	6
	1/100									192	1
AML-05	1	+	24.08	0.03	3	24	3	24	4	24	3
	1/10					24	0.42	24	1	24	0.5
AML-08	1	-	N/A	N/A	N/A	-	N/A	-	N/A	-	N/A
AML-09	1	+	72.18	10.01	91	72	91	72	95	72	94
	1/10					72	5	72	13	72	13
	1/100					72	0.4	72	1	72	1
AML-12	1	+	24	0.22	18	24	18	24	21	24	21
	1/10									24	2
	1/100									24	0.2
AML-18	1	+	21	18.03	95	21	95	21	96	21	96
	1/10					21	7.17			21	14
	1/100					21	0.76			21	1
AML-58	1	-	N/A	N/A	N/A	-	N/A	-	N/A	-	N/A

Measured allele frequencies are highly similar between the assays, with R^2 values of linear fit ranging from 0.77 to 0.99 (Figure 1). The lowest R^2 values are observed when comparing results in amplicon-based versus hybridization-based assays due to the difference in amplification bias between the two methods. A study conducted with an additional 31 AML samples indicated excellent linear fit between the CE-based assay and the NGS-amplicon based assay, with an R^2 value of 0.999 (Figure 2).

Figure 1. Concordance between methods (R^2)

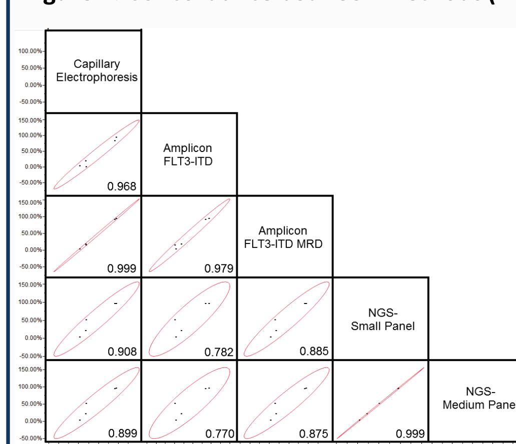
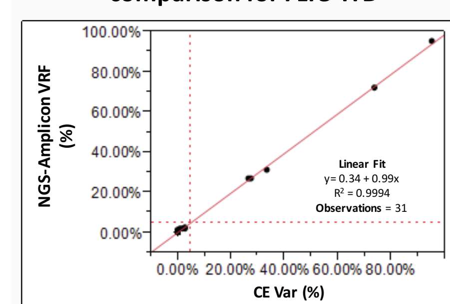


Figure 2. NGS-Amplicon by CE comparison for *FLT3*-ITD



CE Var (%): Percentage of variant converted from signal ratio

Results: Single Nucleotide Variant Detection

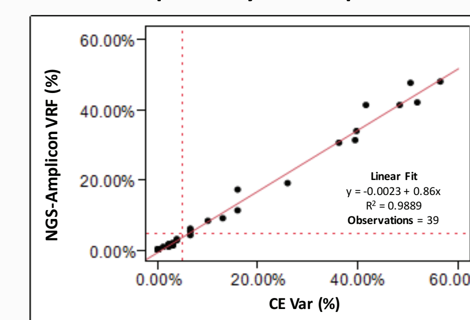
We compared performance across the three assays of a single nucleotide variant in the tyrosine kinase domain of *FLT3* (*FLT3*-TKD). Two clinical samples were positive for the mutation (as determined by CE) and were detected by all three assays (Table 3). As with the other variants, NGS-panel assays detected lower frequency variants that were not detected by the CE assay at frequencies of 10^{-2} and 10^{-3}.

Table 3. Variant: *FLT3*-TKD

Sample Name	Dilution	CE Data	NGS -Amplicon		NGS-Panel Small-Scale		NGS-Panel Medium-Scale	
		Call	Variant	VRF (%)	Variant	VRF (%)	Variant	VRF (%)
AML-03	1	-	-	N/A	-	N/A	-	N/A
AML-04	1	-	-	N/A	-	N/A	-	N/A
	1/10				-	N/A	-	N/A
	1/100						-	N/A
AML-05	1	+	D835E	1.1	D835E	1.3	D835E	1.1
			D835Y	1.3	D835Y	1.3	D835Y	1.3
AML-08	1	-		N/A	-	N/A	-	N/A
AML-09	1	-	-	N/A	-	N/A	-	N/A
	1/10				-	N/A	-	N/A
	1/100				-	N/A	-	N/A
AML-12	1	-	-	N/A	-	N/A	-	N/A
	1/10						-	N/A
	1/100						-	N/A
AML-18	1	+	D835V	28.1	D835V	30.2	D835V	28.1
			D835Y	17.3	D835Y	16.9	D835Y	17.1
	1/10						D835V	4.8
							D835Y	2.6
	1/100						D835V	0.7
							D835Y	N/A
AML-58	1	-			-	N/A	-	N/A

Additionally, 39 clinical AML samples with varying TKD variant allele frequencies (2%-56%; as detected by the CE-based CDx assay) were tested using the NGS-amplicon based FLT3-TKD assay. The NGS assay correlates with excellent linearity when compared with the CE assay (Figure 3).

Figure 3. NGS-Amplicon by CE comparison for *FLT3*-TKD



Conclusions

The ever-changing landscape of drug and molecular diagnostics development requires the diligent application of both cost-effective and scale-appropriate assays. Invivoscribe® assays offer excellent detection and frequency concordance of clinically actionable variants in this set of AML clinical samples, demonstrating reliable variant analysis across assays of different methodologies (CE and NGS) and sensitivities. These assays could be used interchangeably as required for different stages of drug development or clinical treatments. Coupling flexibility of scale (with the associated differences in cost and specimen requirements) with reliability of detection is of critical importance for the evaluation and improvement of patient outcomes and personalized medicine.

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