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Validation of an NGS based assay for monitoring *FLT3* ITD and TKD variants in AML subjects

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Introduction

Internal tandem duplication (ITD) and tyrosine kinase domain (TKD) variants are clinically relevant variants in the FMS-like tyrosine kinase 3 (*FLT3*) gene that can independently promote constitutive activation of the FLT3 kinase. *FLT3* mutations occur in approximately 30% of all Acute myeloid leukemia (AML) patients and are indicative of poor prognosis. AML patients who test positive for *FLT3* mutations have prolonged overall and event-free survival when tyrosine kinase inhibitors, such as midostaurin and gliteritinib fumarate are utilized during treatment. Invivoscribe currently has an FDA approved (USA), MHLW approved (Japan), CE-marked Leukostrat® CDx *FLT3* Mutation Assay, which is used to identify ITD and/or TKD variants. Leukostrat® CDx is a capillary electrophoresis assay that uses DNA extracted from peripheral blood or bone marrow mononuclear cells to detect *FLT3* mutations with a clinical cutoff of 0.05 signal ratio (SR; equivalent to 4.76% variant allele frequency, VAF). Here we describe the development and validation of a simple next-generation sequencing *FLT3* ITD TKD MiSeq Assay with high concordance to Leukostrat® CDx assay that is able to detect *FLT3* ITD and TKD variants quantitatively down to at least 0.5% VAF.

Materials and Methods

Cell line and clinical sample DNA were previously assayed for *FLT3* mutations using the Leukostrat® CDx assay. A panel of contrived samples was generated by diluting genomic DNA with known ITD and/or TKD variants from both clinical and cell line sources into background genomic cell line DNA with no variants. Contrived and clinical samples were used to generate *FLT3* ITD and TKD assay libraries via PCR amplification. Libraries were then pooled and sequenced. Sequencing data was analyzed using proprietary Invivoscribe software.

Limit of blank (LoB), limit of detection (LoD), and linearity were demonstrated using 52 replicates of negative samples and 48 replicates of samples with varying mutant input from cell lines for ITD and TKD.

Precision and Reproducibility were assessed using replicates of both cell line and clinical samples with ITDs ranging from 21 to 126 base pairs and with 3 different TKD mutations.

38 clinical samples were used for clinical concordance. All 38 were used for *FLT3* TKD NGS and CDx assays, while only 29 were used for *FLT3* ITD NGS and CDx assays.

Results: LoD, LoB, and Linearity

LoB

A total of 52 replicates of samples with no *FLT3* mutations were run using both the ITD and TKD assays to calculate LoB. The mean variant read frequencies (VRF) for these samples were 0% for ITD mutations and 0.1% for TKD mutations (Table 1 and Table 2).

LoD

Replicates of contrived samples were run on the ITD and TKD assays from 100% expected VAF down to 0.5% expected VAF for ITD and from 50% expected VAF down to 0.5% expected VAF for TKD. All replicates were detected, thus the LoD is 0.5% for both the ITD and TKD variants (Table 1 and Table 2).

Table 1: VRF of contrived and negative samples determined by *FLT3* ITD Assay

FLT3 ITD					
Expected VAF (%)	N	Mean(%)	Min(%)	Max(%)	CV(%)
0.00	52	0.00	0.00	0.00	--
0.50	6	0.50	0.40	0.49	8.39
1.00	9	0.90	0.79	0.95	6.49
4.00	9	3.80	3.57	4.03	3.74
7.00	9	6.00	5.65	6.27	3.16
10.00	9	8.60	8.37	8.89	1.95
50.00	3	45.80	44.97	46.48	1.70
100.00	3	99.99	99.99	99.99	0

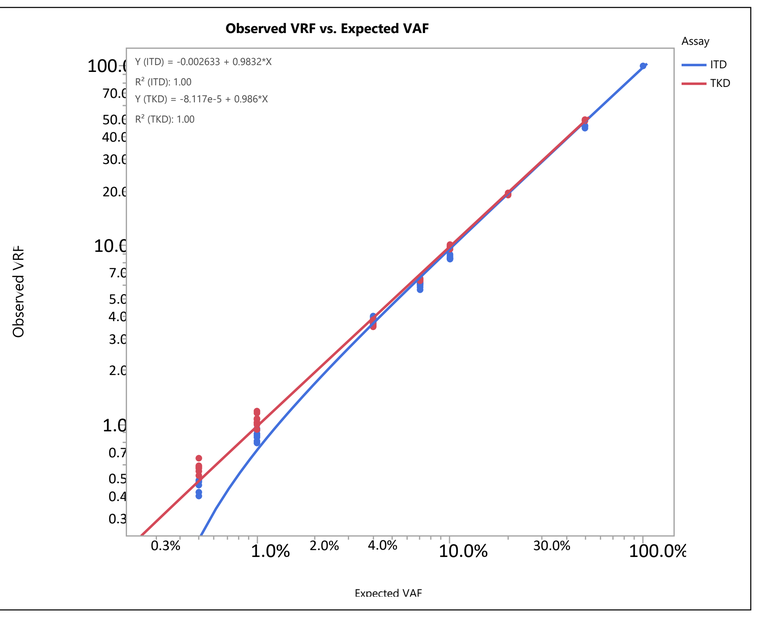
Table 2: VRF of contrived and negative samples determined by *FLT3* TKD Assay

FLT3 TKD					
Expected VAF (%)	N	Mean(%)	Min(%)	Max(%)	CV(%)
0.00	52	0.10	0.09	0.13	8.75
0.50	6	0.60	0.52	0.65	7.57
1.00	9	1.10	0.94	1.19	7.29
4.00	9	3.70	3.51	3.87	3.66
7.00	9	6.40	6.34	6.55	1.06
10.00	9	9.70	9.51	10.09	2.14
20.00	3	19.30	19.04	19.59	1.43
50.00	3	49.80	49.38	50.19	0.81

Linearity

The contrived samples used to generate data for LoD were then assessed for linearity. A log/log graph of linearity is displayed in Figure 1. The ITD and TKD results are highly linear with equations very close to y=x, and R^2 values of 1.00.

Figure 1: Observed VRF values of contrived and negative samples for *FLT3* ITD and *FLT3* TKD graphed against the expected VAF demonstrate highly linear results down to 0.5% for both ITD and TKD.



Results: Precision and Reproducibility

Precision and Reproducibility were performed using DNA from samples with known ITD and TKD mutations diluted into background DNA from a cell line with neither of the representative mutation types. Low negatives (LN, 1% VAF), high negatives (HN, 3.5 – 4.5%), low positives (LP, 5 – 7%), and moderate positives (MP, 10%) samples were run. For the *FLT3* ITD assay, one clinical sample and two cell lines were used with ITD lengths ranging from 21 to 126 bp. For the *FLT3* TKD assay, two clinical samples and one cell line were used. %CV values for samples containing mutations range from 1.98% to 9.86%, while those for negative samples were N/A for the ITD assay (all negative replicates had 0 mutant reads), and 10.86% for the TKD assay.

Variance Components analysis was used to estimate variability of operator, run day (testing with *FLT3* ITD TKD MiSeq Assay), instrument (MiSeq), and random error, expressed in standard deviations, and is summarized in Table 3. In Summary:

- The greatest sources of variability are run day and random effects. The main exceptions to this rule are the Low Negatives for the 126 bp ITD cell line and Clinical Sample A.
- Variation due to instrument was close to zero for all cell lines and dilution levels except for 30 bp ITD cell line MP level (5.8%) and 126 bp ITD cell line HN, LP, and MP levels (2.0%, 10.1%, and 6.8% respectively).

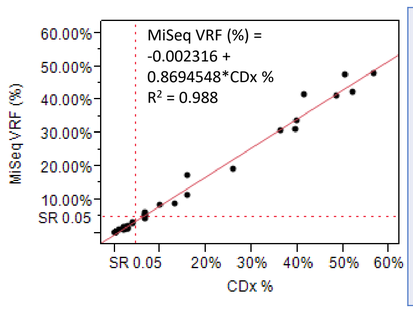
Table 3: VRF Components of Variance per Mutation Type and Dilution Level

Sample	FLT3 Mut	Level	N	Average	Variation due to				Total Variation	
					Operator	Day	MiSeq	Random Error	SD	%CV
					SD(%)	SD(%)	SD(%)	SD(%)		
Clinical Sample A	ITD 21bp	LN	14	1.70E-07	4.2e-8 (23.5%)	1.1e-7 (63.6%)	0.000 (0.0%)	2.3e-8 (12.9%)	0.0004	5.4
		HN	28	3.665	0.000 (0.0%)	0.144 (76.6%)	0.024 (2.0%)	0.076 (21.3%)	0.165	4.2
		LP	28	7.335	0.000 (0.0%)	0.408 (78.3%)	0.000 (0.0%)	0.215 (21.7%)	0.461	5.9
		MP	28	10.478	0.000 (0.0%)	0.103 (17.1%)	0.000 (0.0%)	0.227 (82.9%)	0.25	2.4
30 bp ITD cell line	ITD 30bp	LN	28	2.10E-07	0.000 (0.0%)	2.5e-8 (11.8%)	0.000 (0.0%)	1.9e-7 (88.2%)	0.0005	5.1
		HN	28	3.826	0.000 (0.0%)	0.073 (51.6%)	0.000 (0.0%)	0.071 (48.4%)	0.102	2.5
		LP	28	6.017	0.000 (0.0%)	0.072 (22.3%)	0.000 (0.0%)	0.136 (77.7%)	0.154	2.5
		MP	28	8.814	0.000 (0.0%)	0.162 (71.7%)	0.046 (5.8%)	0.091 (22.5%)	0.192	2
126 bp ITD cell line	ITD 126bp	LN	14	6.60E-08	2.7e-8 (41.7%)	2.1e-8 (26.8%)	0.000 (0.0%)	1.8e-8 (26.8%)	0.0002	9
		HN	28	2.471	0.000 (0.0%)	0.000 (0.0%)	0.000 (0.0%)	0.227 (100.0%)	0.227	9.2
		LP	28	10.161	0.000 (0.0%)	0.295 (30.9%)	0.168 (10.1%)	0.407 (59.0%)	0.53	4.8
		MP	28	20.277	0.000 (0.0%)	1.302 (74.8%)	0.393 (6.8%)	0.647 (18.4%)	1.506	6.1
Clinical Sample B	TKD I836	LN	30	6.4 e-7	0.000 (0.0%)	4.5e-7 (71.6%)	0.000 (0.0%)	1.8e-7 (28.4%)	0.0007	10.1
		HN	30	3.326	0.000 (0.0%)	0.115 (64.7%)	0.000 (0.0%)	0.085 (35.3%)	0.143	4.1
		LP	30	7.261	0.000 (0.0%)	0.257 (69.2%)	0.000 (0.0%)	0.171 (30.8%)	0.309	4
		MP	30	10.661	0.000 (0.0%)	0.272 (60.3%)	0.000 (0.0%)	0.221 (39.7%)	0.35	3.1
D835Y cell line	TKD D835	LN	28	1.70E-07	0.000 (0.0%)	0.000 (0.0%)	0.000 (0.0%)	1.7e-07 (100%)	0.0004	3.9
		HN	42	3.598	0.000 (0.0%)	0.078 (17.0%)	0.000 (0.0%)	0.172 (83.0%)	0.189	5.2
		LP	42	6.448	0.000 (0.0%)	0.089 (23.7%)	0.000 (0.0%)	0.159 (76.3%)	0.182	2.8
		MP	28	9.623	0.000 (0.0%)	0.020 (0.4%)	0.000 (0.0%)	0.301 (99.6%)	0.301	3.1

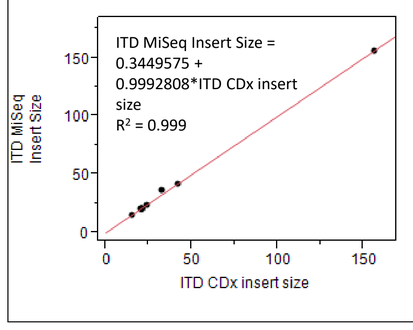
Results: Clinical Concordance

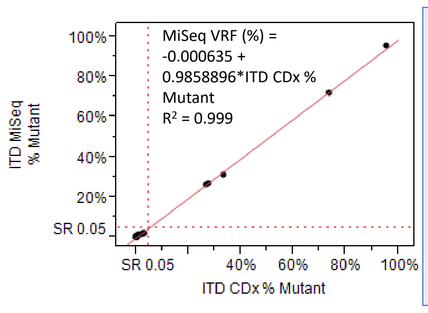
Clinical concordance was performed on 29 samples for *FLT3* ITD and 38 samples for *FLT3* TKD. VRF data is graphed along with ITD insert size data with PPA, NPA, and OPA in Figure 2. There was only one discordant call. This call was for a *FLT3* TKD that was near the cutoff region for the CDx assay (0.05 signal ratio = 4.76%)

Figure 2: Clinical concordance between FLT3 CDx assay and the FLT3 ITD TKD reference assay is very good



FLT3 TKD	Concordance	FLT3 ITD NGS Positive	FLT3 ITD NGS Negative
	CDx Positive	16	1
	CDx Negative	0	21
	PPA= 94.1%	NPA= 100%	OPA= 97.4%





FLT3 ITD	Concordance	FLT3 ITD NGS Positive	FLT3 ITD NGS Negative
	CDx Positive	5	0
	CDx Negative	0	24
	PPA= 100%	NPA= 100%	OPA= 100%

Conclusions

NGS based methodology allows for lower LoB (0% for ITD, 0.1% for TKD) and LoD (0.5% for both ITD and TKD) with highly concordant results compared to capillary electrophoretic detection of *FLT3* ITD and TKD mutations. The *FLT3* ITD TKD MiSeq Assay provides a sensitive method for detecting variants in AML subjects.

AMP Global, May 16-18 2019, Hong Kong