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Next-Generation Sequencing of *FLT3/ITD* for Minimal Residual Disease Monitoring in Leukemia Patients

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Introduction

Minimal residual disease (MRD) detection in patients with leukemia has proven to be useful in the clinical management of disease and can facilitate the development of new therapies. Mutations in fms related tyrosine kinase 3 (*FLT3*) gene are the most common mutations found in acute myeloid leukemia (AML) and are characterized by an aggressive phenotype with a high prevalence of relapse. Internal tandem duplication (ITD) mutations within the juxtamembrane domain are the most common mutations in the *FLT3* gene. The development of a sensitive and specific assay for *FLT3*/ITD mutations represents a significant advancement in guiding treatment decisions.

Materials and Methods

The next-generation sequencing (NGS) MRD assay was designed to target exons 14 and 15 of the *FLT3* gene with a single PCR amplification. Amplicons from up to 24 samples were purified, pooled and sequenced before being analyzed using proprietary software developed by Invivoscribe. Validation was carried out by spiking in fixed amounts of mutant DNA into wild-type DNA to establish a sensitivity equivalent to detection of at least one ITD-containing cell out of 10,000. The DNA input of the assay was 700 ng (>100,000 cell equivalent). The assay was applied to bone marrow DNA from patients with *FLT3*/ITD AML.

Barcode crossover is defined as errors introduced into the DNA barcode from oligo synthesis, PCR, or sequencing causing one barcode sequence to become another barcode sequence. These errors are introduced at ~$1x10^{-4}$ frequency. MiSeq instrumentation also has up to a ~1% rate of run to run DNA carryover which presents a problem for MRD level detection. We have designed our assay with a proprietary approach to reduce the background errors below our $5x10^{-5}$ detection limit.

Results: Sensitivity and Specificity

The sensitivity/specificity and precision/reproducibility of the *FLT3*/ITD MRD assay were demonstrated by testing DNA from two cell lines diluted into a background DNA from a wild type *FLT3* cell line. The validation was carried out with different operators and instruments and conducted on different days. Validation data for samples with read frequency of $1x10^{-4}$ and $5x10^{-5}$ (LOD) of the assay is shown in Table 1. The results show excellent sensitivity/specificity and precision /reproducibility.

Table 1. Summary of sensitivity and specificity

Cell Line	ITD Size (bp)	Expected Frequency	Sample Size	TP	FP	TN	FN	Sensitivity	Specificity
A	30	1.0×10^{-4}	36	36	0	N/A	0	100.0%	N/A
		5.0×10^{-5}	68	68	0	N/A	0	100.0%	N/A
B	126	1.0×10^{-4}	36	35	0	N/A	1	97.2%	N/A
C	N/A	0.0	38	N/A	0	38	N/A	N/A	100.0%

TP: true positive; FP: false positive; TN: true negative; FN: false negative

Results: LOD LOB and Linearity

As shown in Table 2, the limit of detection (LOD) for cell line A (30bp ITD) and cell line B (126 bp ITD) were determined to be at $2.5x10^{-5}$ and $1.0x10^{-4}$, respectively. This was concordant with the fact that ITDs of a certain sequence and at longer lengths are harder to detect by PCR-based assays. There was no ITD detected in the negative cell line (cell line C) indicating that the limit of blank (LOB) is zero. There was no amplicon (ITD positive or negative) detected in the no template control (NTC) samples.

Table 2. Determination of the LOD and LOB of the assay

Cell Line	ITD Size (bp)	Expected Frequency	Sample Size	ITD Detection		% Positive†
				Positive	Negative	
A	30	1.0×10^{-4}	36	36	0	100.0%
		5.0×10^{-5}	68	68	0	100.0%
		2.5×10^{-5}	36	35	1	97.2%
B	126	1.0×10^{-5}	36	35	1	97.2%
C	N/A	0.0	38	0	38	0.0%
NTC	N/A	0.0	34	0	0	0.0%

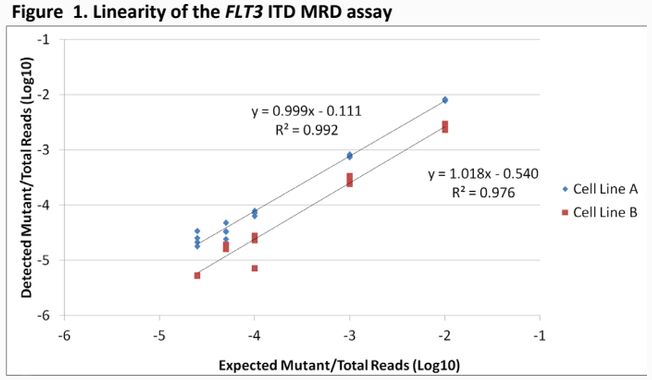
† A positive call requires the detection of a minimum of 3 NGS reads containing a *FLT3* ITD mutation.

DNA from two cell lines with known ITD (30 bp and 126 bp, respectively) were serially diluted into a background DNA from a wild-type *FLT3* cell line and tested with the *FLT3*/ITD MRD assay. Input DNA quantity was 700 ng per dilution point. The experimental data was presented in Table 3 and plotted in the Figure 1. As shown in Figure 1, the linearity of the assay is excellent in the range of $10^{-2} - 10^{-5}$.

Table 3. Determination of the linearity of the assay

Expected Frequency	Detected Frequency	
	Cell Line A	Cell Line B
1.0×10^{-2}	8.2×10^{-3}	3.0×10^{-3}
	8.1×10^{-3}	2.6×10^{-3}
	7.8×10^{-3}	2.7×10^{-3}
	8.3×10^{-3}	2.3×10^{-3}
1.0×10^{-3}	7.5×10^{-4}	2.7×10^{-4}
	7.8×10^{-4}	2.5×10^{-4}
	8.2×10^{-4}	3.3×10^{-4}
	7.4×10^{-4}	2.5×10^{-4}
1.0×10^{-4}	7.8×10^{-5}	7.2×10^{-6}
	6.3×10^{-5}	2.3×10^{-5}
	7.3×10^{-5}	2.8×10^{-5}
	7.2×10^{-5}	2.6×10^{-5}
5.0×10^{-5}	4.8×10^{-5}	1.9×10^{-5}
	3.3×10^{-5}	1.6×10^{-5}
	2.4×10^{-5}	1.7×10^{-5}
	3.3×10^{-5}	0.00
2.5×10^{-5}	2.5×10^{-5}	5.2×10^{-6}
	3.4×10^{-5}	5.3×10^{-6}
	2.1×10^{-5}	0.00
	1.8×10^{-5}	0.00

Figure 1. Linearity of the *FLT3* ITD MRD assay



Results: Clinical Sample Testing

A total of 16 clinical samples tested to be negative for *FLT3*/ITD by the standard PCR assay previously were re-tested by the MRD assay. The investigator conducting the MRD assay was blind to any information regarding the presence or absence of *FLT3*/ITD mutation, its length, or the mutant-to-wild type allelic ratio. The summary of clinical sample testing by standard PCR assay and the MRD assay is shown in Table 4. The MRD assay correctly detected the ITD size in 9 clinical samples. Six patients without detectable *FLT3*/ITD by the MRD assay are disease free.

Table 4. Summary of clinical sample testing by standard PCR assay and the MRD assay

Sample Number	Standard *FLT3*/ITD PCR assay			*FLT3*/ITD MRD assay of follow-up samples		Follow-up Samples
	Diagnostic Samples		Follow-up Samples	Detected ITD Size (bp)	Detected ITD Frequency	
	ITD Size (bp)	Allelic Ratio				
1	33	1490%	Neg	33	1.4×10^{-6}	On treatment
2	48	245%	Neg	48	1.7×10^{-4}	Unavailable
3	69	1%	Neg	69	1.1×10^{-4}	Died
4	24	59%	Neg	24	2.0×10^{-4}	On treatment
5	72	17%	Neg	72	2.8×10^{-5}	On treatment
6	21	1%	Neg	21	4.0×10^{-6}	Relapsed
7	15	11%	Neg	15	1.4×10^{-5}	On treatment
	39	124%		39	3.3×10^{-4}	
8	45	Unavailable	Neg	N/A	0.0	Disease free
9	36	Unavailable	Neg	No PCR amplification		Disease free
10	78	110%	Neg	N/A	0.0	Disease free
11	96	Unavailable	Neg	N/A	0.0	Disease free
12	30	9%	Neg	N/A	0.0	Disease free
13	30	646%	Neg	N/A	0.0	Disease free
14	Detected*		Neg	24	3.7×10^{-3}	Relapsed and died
15	Detected*		Neg	18	1.0×10^{-4}	Relapsed and died
16	Detected*		Neg	N/A	0.0	Disease free

*Size & ratio unavailable

Conclusions

The *FLT3*/ITD MRD assay is a highly-specific test, developed with the accompanying bioinformatics software under full ISO13485 design control, which is at least two orders of magnitude more sensitive than current commercially available assays. In addition, the chemistry and bioinformatics software reliably picked up even the larger ITDs missed entirely by other commercial assays. Importantly, the results of clinical samples tested by this MRD assay showed 100% concordance with clinical outcomes. This assay provides a reliable tool to assess MRD in *FLT3*$^{+}$ AML patients. The *FLT3*/ITD MRD test is currently being offered through Invivoscribe's international clinical Laboratories for Personalized Molecular Medicine (LabPMM).