H04

Assessment of Minimal Residual Disease in Patients with Acute Myeloid Leukemia by Monitoring *FLT3* and *NPM1* Mutations

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Introduction

Current understanding of the somatic driver mutations in acute myeloid leukemia (AML) make genetic-based classification of AML essential to properly stratify patients, assess risk, and optimize treatments. Growing evidence supports that the presence of specific-driver mutations at residual disease levels impacts patient outcomes. In order to optimize treatments and intervene early, it is important to identify the emergence or reemergence of these somatic mutations throughout the course of the disease. The most commonly mutated somatic biomarkers helpful in stratification of AML are mutations in the fms related tyrosine kinase 3 (*FLT3*) and the nucleophosmin (*NPM1*) genes. The development of internationally standardized, regulatory-compliant assays that detect *FLT3* and *NPM1* mutations at MRD levels represents a significant advancement in guiding the treatment of AML.

Material and Methods

The NGS *FLT3*/ITD and *NPM1* MRD assays were designed so they could be run separately or together. The *FLT3*/ITD assay targets exons 14 and 15; the *NPM1* assay targets exon 12 of their respective genes. The DNA input for the assays was 700 ng (>100,000 cell equivalents) for MRD detection. Less DNA input (as low as 50 ng) is feasible to detect relatively high mutation frequencies in diagnostic samples. Three controls: positive, negative, and no-template, were included in every test. Up to 24 amplicons from either one or both assays were purified, pooled, and sequenced. The sequencing data was analyzed using proprietary software developed by Invivoscribe. In the combo linearity assay, a *FLT3* library and a *NPM1* library were combined and loaded onto two MiSeq instruments. DNA isolated from bone-marrow aspirates and peripheral blood from AML patients was tested by both assays.

Results: LoD, LoB, and Linearity of Contrived Samples

Practical, cost-effective, standardized tests were developed to simultaneously detect and monitor *FLT3*/ITD and *NPM1* mutations in AML patients in a clinical setting. The LoD, LoB, and linearity of these assays were accessed by running the libraries alone (single assay) or together (combo assay).

For the *FLT3*/ITD MRD assay, DNA from two cell lines with known ITD (30 bp homozygous and 126 bp heterozygous, respectively) were serially diluted into background DNA from a wild-type *FLT3/NPM1* cell line and tested. For the *NPM1* MRD assay, DNA from a cell line with a known 4 bp insertion (heterozygous) was serially diluted into background DNA from the same wild-type *FLT3*/*NPM1* cell line and tested.

The experimental data for the *NPM1* MRD assay is presented in Table 1 and plotted in Figure 1. As shown in Figure 1, the linearity of the assay is excellent reporting in the range of 10^{-2} – 10^{-5}. There is no significant difference between *NPM1* linearity detected by the single assay or the combo assay. The experimental data for the *FLT3*/ITD MRD assay is presented in Table 2 and plotted in Figure 2. As shown in Figure 2, the linearity of the assay is excellent reporting in the range of 10^{-2} – 10^{-5}. There is no significant difference between *FLT3*/ITD linearity detected by the single assay or the combo assay.

The limit of detection (LoD) is $5x10^{-5}$ for both assays. No mutation is detected in the negative sample in either of the assays (data not shown), indicating that the limit of blank (LoB) is zero.

Table 1. Linearity determination of the *NPM1* MRD assay in single and combo format

Expected Mutation/Total Reads	Detected Mutation/ Total Reads	
	Single	Combo
5.00E-01	4.83E-01	4.95E-01
	4.84E-01	4.98E-01
1.00E-02	9.57E-03	9.29E-03
	8.35E-03	7.93E-03
5.00E-03	3.93E-03	3.67E-03
1.00E-03	5.81E-04	5.61E-04
	6.05E-04	5.03E-04
5.00E-04	3.51E-04	3.77E-04
1.00E-04	5.34E-05	3.44E-05
	1.08E-04	1.19E-04
5.00E-05	6.23E-05	6.15E-05
	4.15E-05	2.98E-05
	2.15E-05	1.57E-05
	3.63E-05	2.42E-05
2.50E-05	2.04E-05	2.22E-05
	9.49E-06	1.57E-05
	1.29E-05	1.33E-05
	1.09E-05	1.03E-05
	3.38E-05	2.81E-05
1.00E-05	2.93E-05	1.53E-05
	1.02E-05	1.87E-05
	1.10E-05	1.30E-05

Figure 1. Linearity of the *NPM1* MRD assay in single and combo format

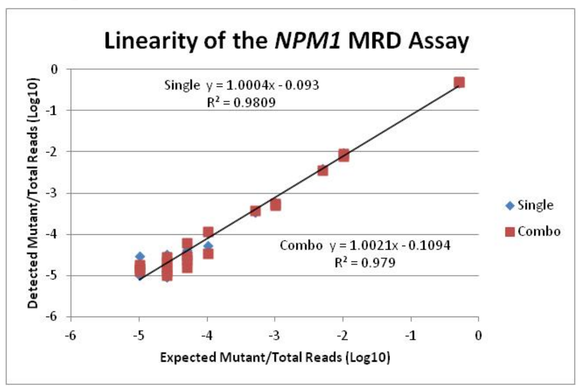
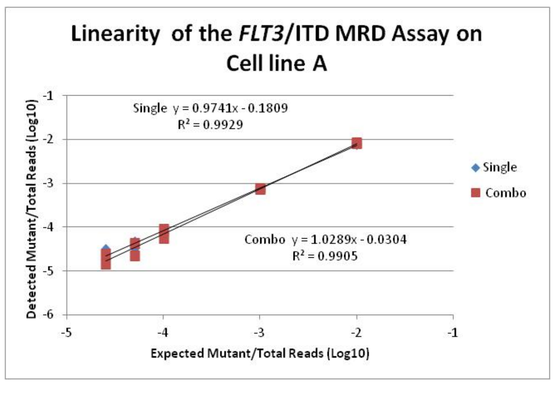
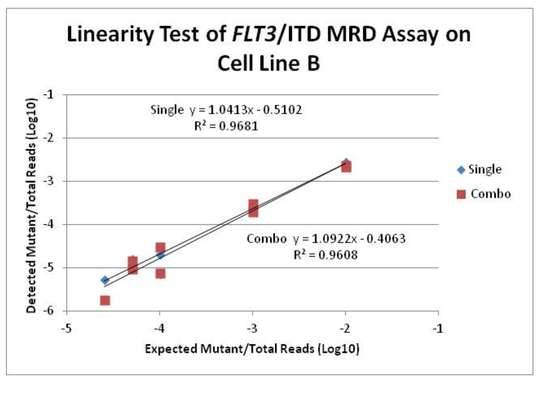


Table 2. Linearity determination of the *FLT3*/ITD MRD assay in single and combo format

Expected Mutation/ Total Reads	Detected Mutation/Total Reads			
	Cell Line A		Cell Line B	
	Single	Combo	Single	Combo
$1.0x10^{-2}$	8.12E-03	8.40E-03	2.82E-03	2.38E-03
	8.02E-03	8.24E-03	2.50E-03	2.17E-03
$1.0x10^{-3}$	7.50E-04	7.40E-04	2.52E-04	3.00E-04
	7.48E-04	7.72E-04	2.44E-04	1.96E-04
$1.0x10^{-4}$	7.48E-05	8.95E-05	7.24E-06	7.67E-06
	6.38E-05	5.76E-05	2.05E-05	3.05E-05
$5.0x10^{-5}$	4.83E-05	4.28E-05	1.62E-05	1.46E-05
	3.34E-05	2.25E-05	1.48E-05	9.61E-06
$2.5x10^{-5}$	2.31E-05	1.47E-05	0.00E+00	0.00E+00
	3.16E-05	2.47E-05	5.30E-06	1.83E-06

Figure 2. Linearity of the *FLT3*/ITD MRD assay in single and combo format





Results: Clinical Sample Testing

MRD tests are more sensitive and specific than commercially available capillary-electrophoresis (CE) assays. As shown in Tables 3, seven *FLT3*/ITD and three *NPM1* mutations were detected in sample AML-04 by the MRD assays. Multiple mutations were also detected in samples AML-05 and AML-12. In addition, nucleotide sequence information was obtained, providing accurate information for tracking and patient follow-up testing.

Table 3. MRD assays are sensitive and specific

Sample ID	*FLT3*/ITD				*NPM1*		
	CE		NGS- MRD		CE	NGS- MRD	
	Size	SR	Size	Mutation/Total Reads		Mutation	Mutation/Total Reads
AML-04	193	0.24	192	1.48E-01	Pos	insTGTA	4.69E-01
			51	5.17E-04			
			24	1.55E-04		insTGCA	3.48E-04
			99	1.42E-04			
			27	4.97E-05		insTCTG	4.30E-06
			81	2.21E-05			
			75	5.52E-06			
AML-05	24.08	0.03	24	2.97E-02	Not Tested	insTCTG	4.06E-01
			33	8.41E-05			
			42	7.51E-06			
AML-12	24	0.22	24	1.78E-01	Pos	insTGCA	4.58E-01
						insTGTA	2.31E-04

A group of samples with clinical outcome information were tested with *FLT3*/ITD and *NPM1* MRD assays. A summary of clinical samples tested by standard CE and MRD assays is shown in Table 4. The MRD assay correctly detected the *FLT3*/ITD and *NPM1* mutations in follow-up samples of patients who were not disease free. Patients without detectable *FLT3*/ITD by the MRD assay were disease free.

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

Sample ID	*FLT3* /ITD					*NPM1*		Patient Status (As of June 2016)
	JH standard *FLT3*/ITD CE assay			IVS *FLT3*/ITD MRD assay of follow-up samples		JH *NPM1* CE assay of Diagnostic sample	IVS *NPM1* MRD assay of follow-up sample (Detected mutant /Total reads)	
	Diagnostic sample		Follow-up sample	ITD Size (bp)	Detected mutant / Total reads			
	ITD size (bp)	Allelic ratio						
JH-1	33	1490%	Neg	33	1.38E-06	Pos	0	On treatment
JH-3	69	~1%	Neg	69	1.11E-04	Pos	1.22E-04	Deceased
JH-6	21	~1%	Neg	21	3.96E-06	Pos	1.57E-05	Relapsed
JH-7	15	11%	Neg	15	1.35E-05	Pos	2.77E-04	On treatment
	39	124%		39	3.30E-04	Pos		
JH-11	96	Unavail.	Neg	N/A	0	Pos	0	Disease free
JH-12	30	9%	Neg	N/A	0	Pos	0	Disease free
JH-13	30	646%	Neg	N/A	0	Neg	0	Disease free
JH-14	Detected (size & ratio unavailable)		Neg	24	3.66E-03	Pos	2.03E-03	Deceased
JH-16	Detected (size & ratio unavailable)		Neg	N/A	0	N/A	0	Disease free

Conclusions

These standardized NGS *FLT3*/ITD and *NPM1* MRD assays are highly specific and at least two orders of magnitude more sensitive than current commercially available capillary-electrophoresis assays. Results of clinical samples tested by these MRD assays showed concordance with capillary-electrophoresis assay results and clinical outcomes. Theses LDT assay services provide reliable and cost-effective tools to assess MRD in AML patients. Developing *FLT3* and *NPM1* MRD mutation assays 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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