invivoscribe

Study of MRD

FLT3 ITD MRD Testing

CLIA/CAP-validated assay

Minimal residual disease (MRD) detection has proven useful in the clinical management of leukemia, as it can be used to better monitor patients, refine treatment, and facilitate the development of new therapies. Mutations in the 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.[1] The most prevalent and clinically significant type of *FLT3* mutation is an internal tandem duplication (ITD) in the juxtamembrane domain.[2] The development of a sensitive and specific assay with design-controlled software that reliably identifies *FLT3* ITD mutations represents a significant advancement in guiding treatment decisions[3].

The Invivoscribe *FLT3* ITD MRD test is a next-generation sequencing (NGS)-based assay developed with cGMP reagents and bioinformatics software following an ISO 13485 and QSR compliant design control system meeting international regulatory requirements required to support a seamless evolution from an LDT service to a registered IVD. The assay has been validated to detect ITDs ranging from 3bp to 126bp in size; however, clinical performance of the assay has demonstrated that ITDs ranging from 3bp to over 200bp in size can be detected. The Invivoscribe *FLT3* ITD MRD assay can detect ITDs even if a diagnostic sample is not available, although testing of a primary sample is preferred in order to identify the specific ITD (length and sequence) to be tracked in subsequent samples. This assay can track *FLT3* ITDs at a sensitivity of 10^{-4} or greater, provided sufficient high quality DNA is available.

References

[1] NEJM. 2013; 368(22): 2059-74

[2] Expert Opin. Ther. Targets. 2015; 19(1): 37-54

[3] Blood Adv. 2018; 2(8): 825-831

1| *FLT3* ITD MRD Clinical Testing Service

The *FLT3* ITD MRD Test developed by Invivoscribe is an amplicon-based NGS assay. This assay can detect mutations with a mutant cell sensitivity of 10^{-4} (1 mutant cell in a background of ten thousand normal cells; equivalent to an allelic sensitivity of $5x10^{-5}$ when a single mutant allele is present).

Recommended sample types include peripheral blood, bone marrow, or high-quality extracted and purified genomic DNA (quantified with a method specific for double-stranded DNA). It is especially important that DNA be free of PCR amplification inhibitors when a high quantity of DNA (> 500 ng) is required for detection of mutations at low frequencies.

The following three controls are included in every test:

1. A positive control with an *FLT3* mutant allelic concentration of $5x10^{-5}$ (a mutant cell concentration of approximately 10^{-4}).
2. A negative control with a wild-type *FLT3* gene.
3. A no template control with water in place of the DNA sample in the PCR reaction.

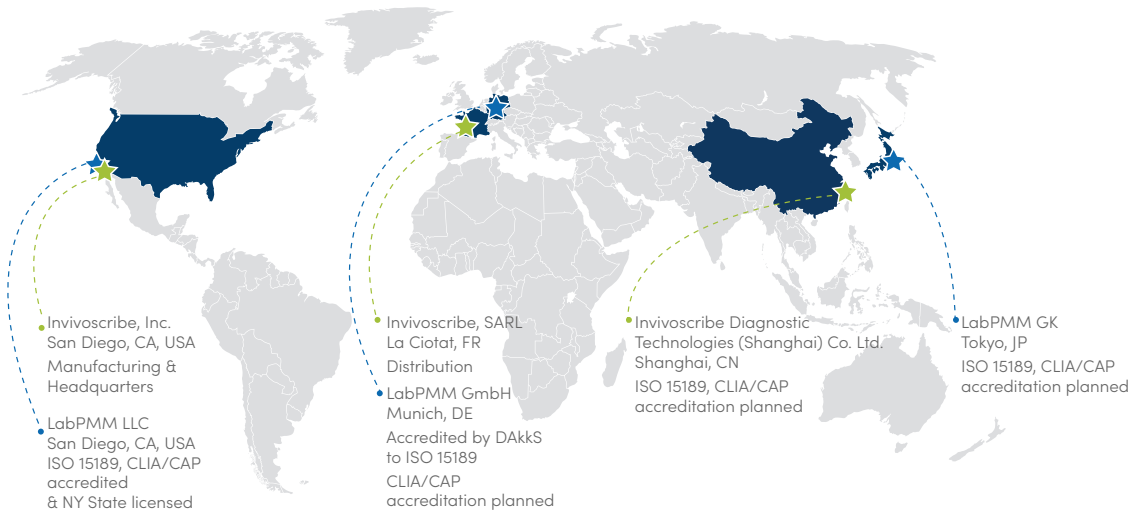
The sequencing output data is analyzed using an Invivoscribe developed proprietary *FLT3* ITD MRD Data Analysis software.

FLT3 ITD MRD testing is currently being provided as a CLIA-validated service through an Invivoscribe fully-owned subsidiary, Laboratories for Personalized Molecular Medicine (LabPMM).

Service description:

Interpretation	Turn - around Time	Specimen Requirements	Shipping Conditions	Storage Conditions
An interpretive report will be issued indicating whether *FLT3* ITD MRD was detected.	7 to 10 business days	• 5 mL of peripheral blood in EDTA or ACD • 3 mL of bone marrow in EDTA or ACD • 1 µg of previously isolated DNA	• Ambient or Cool; Do not freeze	• Room Temp up to 72 hours • 4°C up to 7 days

LabPMM is a network of internationally harmonized reference laboratories, with locations in:



2| Advantages of NGS-MRD Methodologies

MRD testing by NGS provides the sensitivity and specificity needed to detect the presence of residual disease. This method offers a number of advantages over the alternatives of flow cytometry and allele–specific oligonucleotide PCR.

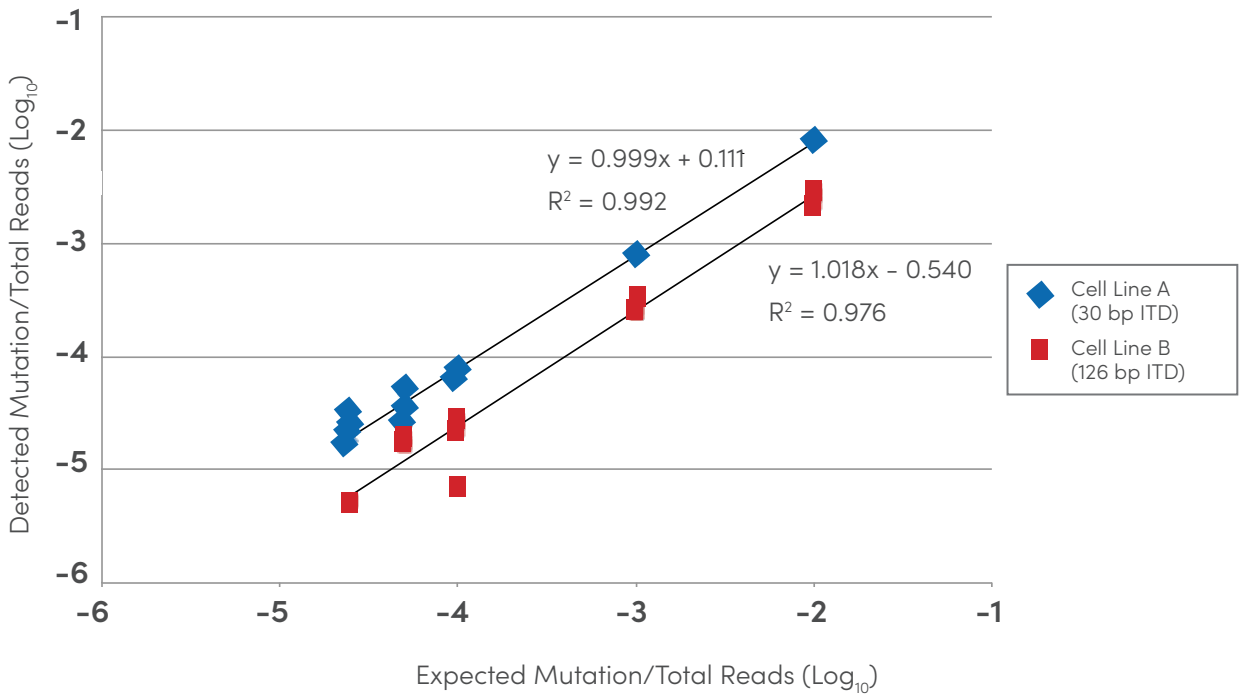
These advantages include the ability to:

1. Standardize the workflow and testing in a regulated environment.
2. Detect clones and newly emerging clones or subclones in follow-up samples.
3. Test at a level of sensitivity only limited by the amount of input DNA interrogated.
4. Generate concordant, internationally harmonized sequence-specific results for mutant detection, as well as confidence lebel calling for negative specimens.

3| Linearity of the Assay

DNA from two cell lines with known *FLT3* ITD mutations (30 bp and 126 bp, respectively) were serially diluted into 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 Invivoscribe *FLT3* ITD MRD software was used to analyze the data **(Table 1)**. As shown below **(Figure 1)**, the assay has excellent linearity for the mutation to total read ratio in the range of 10^{-2} – 10^{-5}.

FIGURE 1: LINEARITY OF THE *FLT3* ITD MRD ASSAY[3]



Reference:

[3] Mark J. Levis et al. (2018) A next-generation sequencing–based assay for minimal residual disease assessment in AML patients with *FLT3*-ITD mutations. *Blood Adv* 2(8):825-831.

TABLE 1. LINEARITY OF THE *FLT3* ITD MRD ASSAY

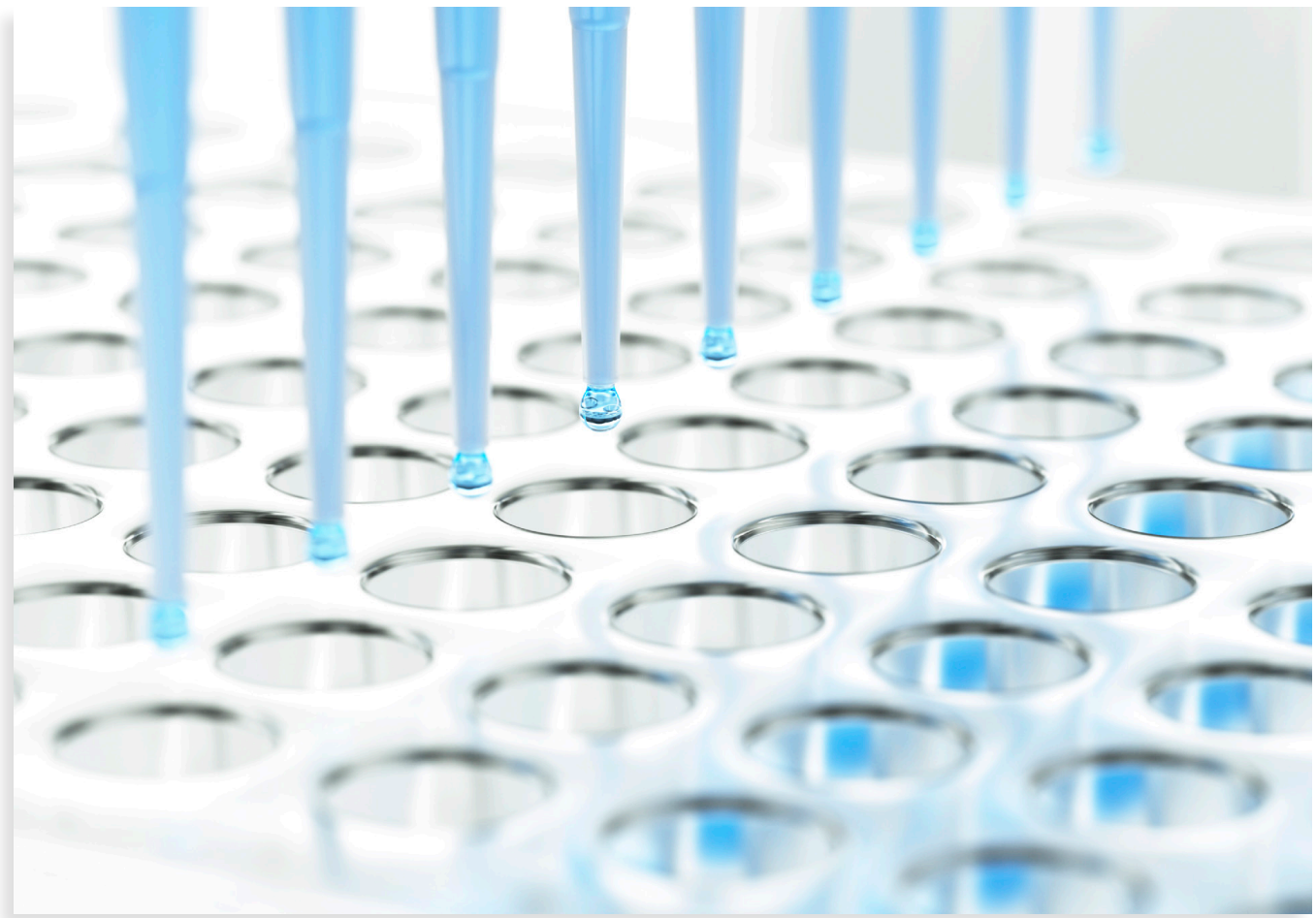
EXPECTED MUTATION/TOTAL READS	DETECTED	
	CELL LINE A (30 bp ITD)	CELL LINE B (126 bp ITD)
	ITD MUTATION/TOTAL READS	ITD MUTATION/TOTAL READS
1.0×10^{-2}	8.23E-03	2.97E-03
	8.10E-03	2.58E-03
	7.80E-03	2.68E-03
	8.31E-03	2.32E-03
1.0×10^{-3}	7.50E-04	2.69E-04
	7.82E-04	2.46E-04
	8.22E-04	3.33E-04
	7.39E-04	2.45E-04
1.0×10^{-4}	7.83E-05	7.16E-06
	6.28E-05	2.29E-05
	7.26E-05	2.76E-05
	7.16E-05	2.62E-05
5.0×10^{-5}	4.79E-05	1.92E-05
	3.30E-05	1.60E-05
	2.41E-05	1.71E-05
	3.34E-05	Not Detected
2.5×10^{-5}	2.52E-05	5.24E-06
	3.39E-05	5.29E-06
	2.14E-05	Not Detected
	1.79E-05	Not Detected

4| Sensitivity and Specificity

The sensitivity and specificity of the *FLT3* ITD MRD assay was demonstrated by testing DNA from two cell lines diluted into background DNA from a wild-type *FLT3* cell line. Input DNA quantity was 700 ng per sample. The data was generated by different operators and instruments and conducted on different days. The results show excellent precision and reproducibility (data not shown). Sensitivities and specificity of the *FLT3* ITD MRD assay are highlighted in **Table 2**. This table shows that the assay sensitivity is >95% for both small (30 bp) and large (126 bp) size ITDs. In addition, we observed no false positives in any of our negative samples (cell line C), nor any unexpected ITDs in any of the positive samples. This points to a very high specificity and positive predictive agreement for the assay.

TABLE 2: SENSITIVITY AND SPECIFICITY OF THE OF THE *FLT3* ITD MRD ASSAY

SAMPLE	ITD SIZE (bp)	EXPECTED FREQUENCY	SAMPLE SIZE	TRUE POSITIVE	FALSE POSITIVE	TRUE NEGATIVE	FALSE NEGATIVE	SENSITIVITY	SPECIFICITY
CELL LINE A	30	1.0×10^{-4}	36	36	0	N/A	0	100.00%	N/A
		5.0×10^{-5}	68	68	0	N/A	0	100.00%	N/A
CELL LINE B	126	1.0×10^{-4}	36	35	0	N/A	1	97.22%	N/A
CELL LINE C	N/A	0.0	38	N/A	0	38	N/A	N/A	100.00%



5| Feasibility Study

A group of samples with clinical outcome information were tested with the *FLT3* ITD MRD assay and a capillary electrophoresis (CE) method by John Hopkins[3]. A summary of clinical samples tested by standard CE and the *FLT3* ITD MRD assays is shown in **Table 3**. The MRD assay correctly detected the *FLT3* ITD mutations in follow-up samples of patients who were not disease free. Patients without detectable *FLT3* ITDs by the MRD assay were disease free. These results demonstrate that the *FLT3* ITD MRD assay is highly specific, and at least two orders of magnitude more sensitive than current commercially available capillary electrophoresis assays.

TABLE 3: SUMMARY OF CLINICAL SAMPLES TESTED BY A STANDARD METHOD AND THE *FLT3* ITD 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	42	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

*Size & ratio unavailable

Reference:
[3] Mark J. Levis et al. (2018) A next-generation sequencing–based assay for minimal residual disease assessment in AML patients with *FLT3*-ITD mutations. *Blood Adv* 2(8):825–831.

6| *FLT3* ITD MRD Assay as predictor of molecular response

FLT3 ITD mutated patients enrolled in the CHRYSALIS study, who were treated with *FLT3*-inhibitory oral doses of 120mg/day or 200 mg/day gilteritinib, had their molecular response assessed from bone marrow aspirates obtained at baseline and at ≥1 additional time point. *FLT3* ITD and total *FLT3* alleles were quantified using the Invivoscribe *FLT3* ITD MRD assay and used to determine molecular response[3]. A Cox regression model of overall survival (OS) by Kaplan-Meier estimation was used to evaluate the impact of ITD variant allele frequency (VAF) on overall survival. Molecular response was defined as follows:

- Molecular response = ITD VAF (*FLT3* mutant reads:*FLT3* total reads) of $\leq 10^{-2}$
- Major molecular response = ITD VAF of $\leq 10^{-3}$
- Negative MRD status = ITD VAF of $\leq 10^{-4}$

As shown below, patients with molecular response had longer overall survival than those without a molecular response. This is the first demonstration of molecular response to a *FLT3* inhibitor in AML[3].

TABLE 4: SUBJECTS, OVERALL SURVIVAL

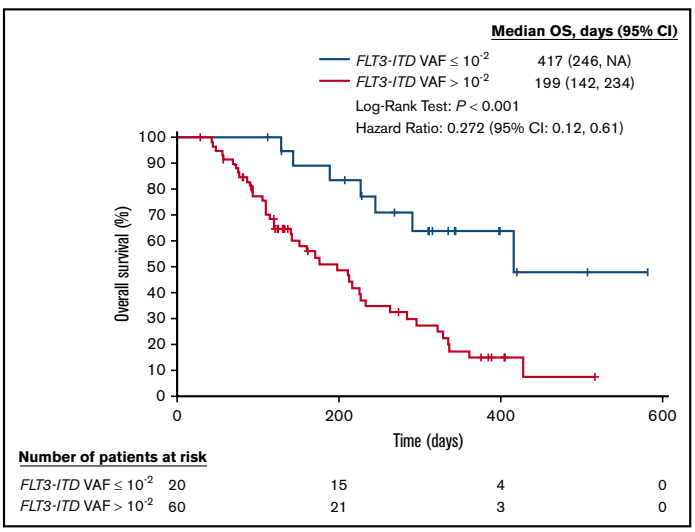
	Achieved a molecular response		Did not achieve a molecular response		
Molecular response	**n**	**Median OS (95% CI), d**	**n**	**Median OS (95% CI), d**	***P***
ITD VAF $\leq 10^{-2}$	20	417 (246–NA)	60	199 (142–234)	<.001
ITD VAF $\leq 10^{-3}$	18	417 (228–NA)	62	213 (143–264)	.003
ITD VAF $\leq 10^{-4}$ (MRD negative)	13	417 (228–NA)	67	213 (144–264)	.002

Comparison between patients achieving a molecular response (*FLT3* ITD VAF $\leq 10^{-2}$, $\leq 10^{-3}$, or negative as defined by ITD VAF $\leq 10^{-4}$) by the MRD assay and those not achieving a molecular response by the MRD assay. The P values were determined by the log-rank test.

Reference:

[3] Mark J. Levis et al. (2018) A next-generation sequencing–based assay for minimal residual disease assessment in AML patients with *FLT3*-ITD mutations. *Blood Adv* 2(8):825-831.

FIGURE 2: SUBJECTS, OVERALL SURVIVAL STRATIFIED BY MOLECULAR RESPONSE, USING THE INTERNATIONALLY-HARMONIZED *FLT3* ITD MRD ASSAY (10^{-2})



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The Invivoscribe *FLT3* ITD MRD Assay is a sensitive, specific, and prognostically relevant assay for the detection of MRD in *FLT3* ITD mutated AML patients. It can be used as a surrogate endpoint - reducing clinical trial duration and cost and, most importantly, giving patients access to drugs more quickly.

029 Rev. E May 2018 280370

FLT3 Mutation Testing is performed pursuant to patents licensed from Takara Bio Inc. of Kusatsu (Shiga prefecture), Japan.

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