SUMMARY OF SAFETY AND EFFECTIVENESS DATA (SSED)

I. GENERAL INFORMATION

Device Generic Name: Somatic Mutation PCR test

Device Trade Name: LeukoStrat® CDx FLT3 Mutation Assay

Device Procode: OWD

Applicant's Name and Address: Invivoscribe Technologies, Inc.
6330 Nancy Ridge Drive, Suite 106,
San Diego, CA 92121

Date(s) of Panel Recommendation: None

Premarket Approval Application (PMA) Number: P160040

Date of FDA Notice of Approval: April 28, 2017

Priority Review: Granted priority review status on October 21, 2016, because the device addresses an unmet medical need as demonstrated by significant meaningful advantage.

II. INDICATIONS FOR USE

The LeukoStrat® CDx FLT3 Mutation Assay is a PCR-based, in vitro diagnostic test designed to detect internal tandem duplication (ITD) mutations and the tyrosine kinase domain (TKD) mutations D835 and I836, in the FLT3 gene in genomic DNA extracted from mononuclear cells obtained from PB or BM aspirates of patients diagnosed with acute myelogenous leukemia (AML).

The LeukoStrat® CDx FLT3 Mutation Assay is used as an aid in the selection of patients with AML for whom midostaurin (RYDAPT®) treatment is being considered.

The LeukoStrat® CDx FLT3 Mutation Assay is to be performed only at Laboratory for Personalized Molecular Medicine (LabPMM) LLC, a single laboratory site located at 6330 Nancy Ridge Dr., San Diego, CA 92121.

III. CONTRAINDICATIONS

There are no known contraindications.

IV. WARNINGS AND PRECAUTIONS

The warnings and precautions can be found in the LeukoStrat® CDx FLT3 Mutation Assay labeling.

V. DEVICE DESCRIPTION

The LeukoStrat® CDx FLT3 Mutation Assay (CDx) is an *in* vitro diagnostic device performed in a single laboratory, LabPMM, in San Diego, CA. The assay includes reagents, software, instruments and procedures for testing DNA extracted from mononuclear cells isolated from patient specimens to determine if FLT3 ITDs or TKD mutations are present. The Kit contains sufficient reagents for 150 reactions.

Device Instrumentation and Software
The FLT3 Mutation Assay to be used on serialized Applied Biosystems 3500xL Genetic Analyzers which is configured with FLT3 Mutation Analysis GeneMapper Software, Version 1.5. The serial numbers for the 4 instruments are S/N 21106-231, S/N 23321-020, S/N 23321-030, and S/N 25351-081.

Specimen Preparation
Peripheral blood (PB) specimens (≥1mL) or bone marrow aspirate (BM) specimens (≥0.25mL) are collected in tubes with sodium heparin anticoagulant. (Frozen blood is unacceptable as a sample for testing.) Blood and BM specimens are received on cold packs, the mononuclear cells are separated from the specimens via passage over a Ficoll column, and then counted to ensure that the extraction columns are not overloaded. The DNA is extracted from the mononuclear cells using QIAGEN DSP blood mini kits. After DNA isolation, the DNA is quantified . DNA samples ≥10.5 ng/µL must be diluted to 10 ng/µL in AE buffer.

PCR Amplification and Detection
Fms-like tyrosine kinase 3 (FLT3) is a gene that encodes for a tyrosine kinase that activates pathways in hematopoietic cells. FLT3 ITDs are caused by duplication and insertion of a portion of the FLT3 gene that includes the region in and around the juxtamembrane (JM) region of the FLT3 gene. These mutations vary in both the location and the length of the inserted duplicated DNA sequence. FLT3 TKD mutations detected by the LeukoStrat® CDx FLT3 Mutation Assay are caused by nucleic acid substitutions and/or deletions that result in a change in the EcoRV restriction enzyme cut site. This includes TKD mutations at D835 and I836. The FLT3-ITD and TKD mutations result in constitutive autophosphorylation and activation of FLT3.

FLT3-ITD: The LeukoStrat® CDx FLT3 Mutation Assay uses primers that are in the JM region. The forward and reverse PCR primers are fluorescently labeled with different fluorophores that serve to confirm the presence of sample signal. Wild type FLT3 alleles will amplify and produce a product measured at 327±1 bp as measured by the LeukoStrat® CDx FLT3 Mutation Assay, while alleles that contain ITD mutations will produce a product that exceeds the wild-type result by at least 3 bp. The LeukoStrat® CDx FLT3 Mutation Assay is validated to detect ITD containing amplicons approximately up to 650 bp (i.e., ITDs up to approximately 279 bp).

FLT3-TKD: Wild-type alleles of the FLT3 gene include an EcoRV restriction digest site. When a substitution and/or deletion change occurs, the restriction digest recognition site disappears, and the EcoRV endonuclease is unable to identify and digest the DNA at this site. The LeukoStrat® CDx FLT3 Mutation Assay uses primers that lie on either side of the TKD region. The FLT3 target region is amplified using PCR and then an EcoRV restriction digest is performed. One of the PCR primers is labeled with a fluorophore and the other contains an EcoRV restriction site, so both wild type and mutant alleles are digested. The digestion pattern identifies loss of the normal gene sequence and ensures that digestion occurred. Wild type alleles of the FLT3 gene yield digestion products of 79±1 bp whereas mutant alleles yield products of 125±1 (deletion) or 127±1 (substitution) bp from the original undigested amplicon product of 145±1 (deletion) or 147±1 (substitution) bp as measured by the LeukoStrat® CDx FLT3 Mutation Assay.

Interpretation of Results

Following amplification, the resulting amplicons are analyzed via capillary electrophoresis. The assay measures the ratio of signals from mutation against a background of signal from wild type. The mutant:wild-type signal ratio (SR) is calculated by the FLT3 Mutation Analysis Software and automatically evaluated against the cut-off (medical decision point) of 0.05. The SR is the peak area of the mutant signal, if present, divided by the peak area of the wild-type signal. If the mutant:wild type SR for either ITD or TKD in a valid sample is at or above the cut-off of 0.05, the result will be interpreted and reported as Positive (mutation detected). Below the cut-off, the result will be interpretated and reported as Negative. The mutation status of a sample is defined by the rules below in Table 1.

Table 1. Determination of Sample FLT3 Mutation Status

Scenario	ITD Result	TKD Result	Sample Mutation Status
1	Positive (SR ≥0.05)	Positive (SR ≥0.05)	Positive
2	Negative (SR <0.05)	Negative (SR <0.05)	Negative
3	Invalid	Invalid	Invalid
4	Positive (SR ≥0.05)	Negative (SR <0.05)	Positive
5	Negative (SR <0.05)	Positive (SR ≥0.05)	Positive
6	Positive (SR ≥0.05)	Invalid	Positive
7	Negative (SR <0.05)	Invalid	Invalid
8	Invalid	Positive (SR ≥0.05)	Positive
9	Invalid	Negative (SR <0.05)	Invalid

Controls and Run Validity

Positive Controls

The FLT3-ITD positive control is designed to yield a signal ratio of between 0.05 and 0.11. The control is produced by mixing gDNA from a cell line (MV4-11) containing a FLT3-ITD mutation with DNA from a cell line (Jurkat) without a FLT3 ITD mutation.

The FLT3-TKD positive control is designed to yield a signal ratio of between 0.05 and 0.11. The control is produced by mixing gDNA from a cell line containing a FLT3-TKD

mutation (TVI-TKD cell line) with DNA from a cell line without a FLT3 ITD mutation (Jurkat cell line).

The positive controls are meant to ensure the assay is properly evaluating positive ITD and TKD mutated samples.

No Template Control
Sterile water serves as a negative control to assess if any samples were free from contamination.

Extraction Control
Extraction control is gDNA isolated from a cell line without either a FLT3-ITD or a FLT3-TKD mutation (Jurkat cell line). This control ensures the extraction step functioned properly and works as a negative control. If an Extraction Control does not meet its validity criteria for signal strength, signal ratio, and/or peak size, all samples associated with that Extraction Control will be labeled Fail.

The FLT3 Mutation Analysis Software automatically evaluates the results against the criteria shown in Table 2 for peak size when using the ITD or TKD PCR reaction.

Table 2. Run Validity Criteria

Run Validity Control	ITD Requirements	TKD Requirements
Positive Control	Valid (meets signal strength and peak size specifications) Mutant:wild type signal ratio ≥0.05 and ≤0.11	Valid (meets signal strength and peak size specifications) Mutant:wild type signal ratio ≥0.05 and ≤0.11
No Template Control	No peaks in the region of interest	No peaks in the region of interest

VI. ALTERNATIVE PRACTICES AND PROCEDURES

There are no other FDA approved or cleared alternatives for testing of FLT3 mutations in human specimens.

VII. MARKETING HISTORY

The LeukoStrat® CDx FLT3 Mutation Assay has not been marketed in the United States or any foreign country.

VIII. POTENTIAL ADVERSE EFFECTS OF THE DEVICE ON HEALTH

Failure of the device to perform as expected or failure to correctly interpret test results may lead to incorrect FLT3 mutation results and subsequently, improper patient management decisions in AML treatment. Patients with a false negative assay result may not be considered for treatment with RYDAPT® (midostaurin). Patients with a false

positive assay result may receive treatment with RYDAPT® (midostaurin) for which there is no expectation of benefit. There is also a risk of delayed results, which may lead to delay in treatment.

For the specific adverse events related to midostaurin (RYDAPT®) (midostaurin) that occurred in the clinical studies, refer to Section X below.

IX. SUMMARY OF NONCLINICAL STUDIES

A. Laboratory Studies

1. Correlation to a Reference Method (Accuracy)

All Evaluable Set: The accuracy of the LeukoStrat® CDx FLT3 Mutation Assay was determined by comparing the results of the LeukoStrat® CDx FLT3 Mutation Assay to a validated high throughput sequencing method using specimens from the clinical trial. The samples for the method comparison study were a subset of the FLT3 bridging study samples evaluated by a clinical trial assay (CTA)> the samples included all available and evaluable FLT3 mutation positive (CTA+) samples and approximately 300 FLT3 mutation negative (CTA-) samples. The negative sample subset was selected by a randomization algorithm with the proportion from each CTA laboratory test site matching the proportion from that site in the overall A2301 study. After accounting for specimens with valid results, 505 CTA+ specimens were included and 263 CTA- specimens for a total of 768 patient specimens. Four of these contained low DNA quantity and were tested on deviation. Of the 764 results, 487 were FLT3 positive by both assays and 230 were negative by both as summarized in Table 2. Percent positive agreement (PPA)< negative percent agreement (NPA) and overall agreement (OA) with and without the invalid results are shown in Table 3.

Table 1. Concordance between CDx and High throughput Sequencing for All Samples

CDx*	Sequencing		
	Positive	**Negative**	**Total**
Positive	487	6	493
Negative	31	230	261
Invalid	7	3	10
Total	525	239	764

Table 2. Agreement between CDx and High throughput Sequencing

Agreement Measure	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	**95% CI(1)**	**Percent Agreement (N)**	**95% CI(1)**
PPA	94.0% (487/518)	(91.6%, 95.9%)	92.8% (487/525)	(90.2%, 94.8%)
NPA	97.5% (230/236)	(94.5%, 99.1%)	96.2% (230/239)	(93.0%, 98.3%)
OPA	95.1% (717/754)	(93.3%, 96.5%)	93.8% (717/764)	(91.9%, 95.4%)

Results by Mutation Type

FLT3-ITD: The ITD detected population refers to the samples that harbor only ITD mutations based on Sequencing. Among the 378 ITD samples, 64% showed only one (1) ITD variant with the remaining containing multiple ITD mutations. The ITD insert length ranged from 3 bp to 209 bp. Most samples with ITD mutations were of insert lengths less than 100 bp (>85%). Thirty-seven (37) of the ITD samples contained insert lengths greater than or equal to 100 bp. The size distribution of the ITDs is shown below in Figure 2. The number of specimens (y axis) is shown as a function of the insert size on the Y-axis.

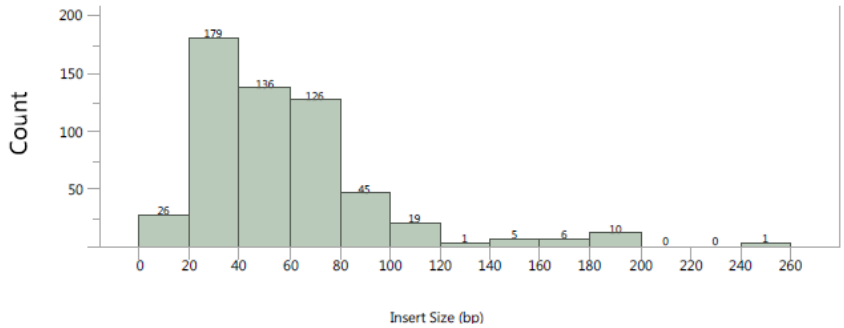


Figure 1: Distribution of ITD Insert Sizes by CDx (381 Positive Samples, N Insert Sizes = 554, Mean=55.6).

Nine patients failed to yield valid ITD results with the CDx. There were 57 discordant results among the 764 samples analyzed. Of the 57 discordant results, 50 showed low variant fraction reads by Sequencing and the CDx reported mutation negative based on the clinical cut-off (SR=0.05). The point estimates of PPA, NPA and OPA are 86.8%, 97.3%, and 91.4% respectively including the CDx invalids (Table 4a). Without the CDx invalids, the PPA, NPA and OPA are at or above 88%.

Table 3a. Agreement on ITD between CDx and High throughput Sequencing for FLT3-ITD results

Agreement Measure	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	95% CI(1)	Percent Agreement (N)	95% CI(1)
PPA	88.0% (375/426)	(84.6%, 91.0%)	86.8% (375/432)	(83.2%, 89.9%)
NPA	98.2% (323/329)	(96.1%, 99.3%)	97.3% (323/332)	(94.9%, 98.8%)
OPA	92.5% (698/755)	(90.3%, 94.2%)	91.4% (698/764)	(89.1%, 93.3%)

FLT3-TKD: The TKD detected population refers to samples that harbor only TKD mutations, based on high throughput sequencing. Among the 94 TKD samples, 79% contained one (1) TKD variant (substitution or deletion) while 20/94 (21%) contained two TKD variants. As expected, the single nucleotide substitution at codon D835 was the predominant mutation, mainly as D835Y. The D835H, D835V

and I836S mutations were also observed at lower prevalence. Thirteen percent (13%) of the TKD positive samples contained the deletion variant at I836 as either a deletion only or deletion plus substitution.

One hundred thirty-seven (92.6%) of 148 samples, identified as positive for a TKD mutation by sequencing, were identified as TKD positive by the CDx assay. Six hundred eleven (98.2%) of the 616 samples identified as TKD negative by sequencing, were TKD negative by the CDx assay. Eight patient sampless yielded an invalid TKD result by the CDx and 8 of the 764 samples tested were discordant. The 8 discordant results showed low variant fraction reads by high throughput sequencing and the SR for the CDx reported mutation negative result found to be below the assay cut-off. Results for TKD agreement with and without invalids are summarized in 4b.

Table 4b. Agreement on TKD between CDx and High throughput Sequencing for FLT3-TKD

Agreement Measure	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	95% CI[(1)]	Percent Agreement (N)	95% CI[(1)]
PPA	94.5% (137/145)	(89.4%, 97.6%)	92.6% (137/148)	(87.1%, 96.2%)
NPA	100.0% (611/611)	(99.4%, 100.0%)	99.2% (611/616)	(98.1%, 99.7%)
OPA	98.9% (748/756)	(97.9%, 99.5%)	97.9% (748/764)	(96.6%, 98.8%)

Results by Specimen Type

Results were analyzed separately for peripheral blood and bone marrow and demonstrated to be comparable. The Results are shown Tables 5a through 5d

Table 5a. Agreement between CDx and High throughput **Sequencing (ITD BM)**

Agreement	CDx ITD Conditional on Sequencing ITD		Sequencing ITD Conditional on CDx ITD	
	% (n/N)	95% CI [(1)]	% (n/N)	95% CI [(1)]
PPA	87.3% (309/354)	83.4%, 90.6%	98.4% (309/314)	96.3%, 99.5%
NPA	98.2% (274/279)	95.9%, 99.4%	85.9% (274/319)	81.6%, 89.5%
OPA	92.1% (583/633)	89.7%, 94.1%	92.1% (583/633)	89.7%, 94.1%

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method

Table 5b. Agreement between CDx and High throughput **Sequencing (ITD PB)**

Agreement	CDx ITD Conditional on Sequencing ITD		Sequencing ITD Conditional on CDx ITD	
	% (n/N)	95% CI (1)	% (n/N)	95% CI (1)
PPA	90.4% (66/73)	81.2%, 96.1%	98.5% (66/67)	92.0%, 100%
NPA	98.0% (49/50)	89.4%, 99.9%	87.5% (49/56)	75.9%, 94.8%
OPA	93.5% (115/123)	89.6%, 97.2%	93.5% (115/123)	89.6%, 97.2%

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method

Table 5c. Agreement between CDx and High throughput **Sequencing (TKD BM)**

Agreement	CDx ITD Conditional on Sequencing ITD		4 Sequencing ITD Conditional on CDx ITD	
	% (n/N)	95% CI (1)	% (n/N)	95% CI (1)
PPA	95.8% (115/120)	90.5%, 98.6%	100% (115/115)	96.8%, 100%
NPA	100% (511/511)	99.3%, 100%	99.0% (511/516)	97.8%, 99.7%
OPA	99.2% (626/631)	98.2%, 99.7%	99.2% (626/631)	98.2%, 99.7%

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method

Table 5d. Agreement between CDx and High throughput **Sequencing (TKD PB)**

Agreement	CDx ITD Conditional on Sequencing ITD		Sequencing ITD Conditional on CDx ITD	
	% (n/N)	95% CI (1)	% (n/N)	95% CI (1)
PPA	88.0% (22/25)	68.8%, 97.5%	100% (22/22)	84.6%, 100%
NPA	100% (99/99)	96.3%, 100%	97.1% (99/102)	91.6%, 99.4%
OPA	97.6% (121/124)	93.1%, 99.5%	97.6% (121/124)	93.1%, 99.5%

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method

2. Analytical Sensitivity

The detection capability of the LeukoStrat® CDx FLT3 Mutation Assay was evaluated.

Limit of blank (LoB)

The limit of blank of the assay was assessed using 100% wild-type DNA from the Nalm6 wild type cell line. The study was conducted with DNA from 66 replicates diluted to 10 ng/µl using the ITD or TKD reaction. Under all conditions, the SR was 0.00 for the ITD assay and between 0.00 and 0.01 in the TKD assay. Thus, the false positive rates are 0% for both ITD and TKD.

Limit of detection (LoD)

LoD of the assay was evaluated in two studies. The first study used contrived samples created by blending cell lines with leukocyte-depleted whole blood. Cell line samples were used to represent three ITD insert sizes: 30 bp insert, 126 bp insert, and a 279 bp insert as described in Table 7. Additional cell lines (TVI-TKD) containing either the D835 mutation or the I836 TKD mutation were also assessed. DNA was diluted to 5 ng/µL, 10 ng/µL, and 15 ng/µL and tested at two allelic ratios for each cell line as shown in the Table 8. Extraction Controls were diluted and tested only at 10 ng/µL as Negative Controls. Each mutation was tested in a set of at least 22 replicates in an effort to achieve a target of >95% of valid results per dilution level. The study was designed to detect the lowest mutant/wild-type allelic ratio (AR) in which a specimen was still called positive because it was above the SR 0.05 that could be detected above the LoB in at least 95% of cases.

The LoD for the LeukoStrat® CDx FLT3 Mutation Assay was 3% for ITD insert of 30 bp, 5% for 126 bp ITD and 33% for 279 bp ITD. The LoD for the TKD mutation at codon D835 was 5%. While the LoD for the larger ITDs was significant, as shown in Figure 1, the majority (95%) of patients had ITDs < 120.

Table 8. Limit of Detection with Cell Lines

FLT3	AR	DNA Dilution	Sample Size	Valid N	Valid N >LoB	% >LoB	Mean SR	Std Dev	Median SR	Min-Max SR	% CV
30 bp ITD	0.03	5 ng/µL	33	22	21	95.5%	0.028	0.007	0.03	0.00 - 0.03	24.7
		10 ng/µL	33	33	33	100.%	0.029	0.003	0.03	0.02 - 0.03	10.0
		15 ng/µL	33	33	33	100.%	0.028	0.004	0.03	0.02 - 0.03	13.9
	0.05	5 ng/µL	33	30	30	100.%	0.036	0.005	0.04	0.03 - 0.04	13.5
		10 ng/µL	33	33	33	100.%	0.037	0.006	0.04	0.03 - 0.05	15.8
		15 ng/µL	33	33	33	100.%	0.035	0.005	0.03	0.03 - 0.04	14.6

D835	0.05	5 ng/µL	33	33	33	100.%	0.020	0.003	0.02	0.01 - 0.03	15.0
		10 ng/µL	33	33	33	100.%	0.020	0.002	0.02	0.01 - 0.02	8.8
		15 ng/µL	33	33	33	100.%	0.020	0.000	0.02	0.02 - 0.02	0.0
	0.08	5 ng/µL	33	33	33	100.%	0.032	0.004	0.03	0.03 - 0.04	13.4
		10 ng/µL	33	33	33	100.%	0.034	0.005	0.03	0.03 - 0.04	14.6
		15 ng/µL	33	33	33	100.%	0.038	0.004	0.04	0.03 - 0.04	11.6
126 bp ITD	0.05	5 ng/µL	22	22	22	100.%	0.043	0.005	0.04	0.04 - 0.05	11.0
		10 ng/µL	22	22	22	100.%	0.039	0.004	0.04	0.03 - 0.04	9.1
		15 ng/µL	22	22	22	100.%	0.030	0.000	0.03	0.03 - 0.03	0.0
279 bp ITD	0.30	5 ng/µL	22	19	19	86.3%	0.091	0.014	0.09	0.08 - 0.14	15.8
		10 ng/µL	22	22	20	90.9%	0.063	0.021	0.07	0.00 - 0.08	34.0
		15 ng/µL	22	22	21	95.5%	0.049	0.013	0.05	0.00 - 0.06	26.6
	0.33 (repeat)	5 ng/µL	22	22	22	100.%	0.118	0.012	0.12	0.09 - 0.13	9.8
		10 ng/µL	22	22	22	100.%	0.117	0.014	0.115	0.09 - 0.14	11.5
		15 ng/µL	22	22	21	95.5%	0.154	0.262	0.10	0.00 - 1.32	170.3

A second study with clinical specimens was conducted to confirm the LoD observations obtained with cell lines. Five clinical samples were diluted with clinical negative samples in order to yield a targeted signal ratio (TSR) within the linear range of an appropriate cell line standard curve. Each specimen was diluted to 5 levels representing a low negative (LN), high negative (HN), near the cut-off (CO), a low positive (LP), and a medium positive (MP). These linear range samples were tested in the FLT3 Mutation Assay and an average SR value was determined. Each clinical LoD sample dilution was tested 20 times for each dilution level over four nonconsecutive days (5 replicates per day) by one operator using one equipment set. The allelic ratio (AR) of each clinical LoD sample dilution was calculated using the AR estimated from the cell line standard curves. The ARs of the clinical LoD samples were estimated based on the study meeting the following acceptance criteria:

- The SR and AR where FLT3 mutations can be detected above the limit of blank (LoB) in ≥95% of replicates (Analytical LoD).
- The AR near the clinical cut-off, a SR of 0.04 – 0.06 (Cut-off).

- The AR and SR that is detected above the clinical cut-off in ≥95% of replicates (Above Cut-off).

The AR is reprentative of multiple peaks and therefore does not directly indicate the percentage of mutant in the sample. Study results show that the assay LoD was determined as 0.144 AR (12.6%) for the I836 mutation, 0.089 AR (8.2%) for the D835 mutation, 0.107 AR (9.7%) for the small ITD, 0.189 AR (15.9%) for the medium ITD, and 0.539 AR (35%) for the large ITD. Results are summarized for each of the 5 dilution levels in Table 9. The %CV for the I836 mutation was 5.7%, 4.5% for the D835 mutation, 7.1% for the 24 bp ITD, 7.1% for the 66 bp ITD, and 25.6% for the 217 bp ITD. Thus, all FLT3 mutations met the acceptance criterion of 25% CV except the large ITD.

Table 9a. SR, AR and LoD per each Sample and Dilution Level

Sample ID	Mutation	Level	TSR	SR Mean	AR of Blend	Valid N	N (%) SR > LoB	N (%) SR > 0.05	*Classification
FPB006	TKD I836	LN	0.02	0.02	0.039	20	20 (100.0)	0	Analytical LoD
		HN	0.03	0.03	0.057	20	20 (100.0)	0	-
		CO	0.05	0.05	0.094	20	20 (100.0)	16 (80.0%)	Cut-off
		LP	0.08	0.07	0.144	20	20 (100.0)	20 (100.0)	Above Cut-off
		MP	0.13	0.12	0.224	20	20 (100.0)	20 (100.0)	-
TBM232	TKD D835	LN	0.02	0.02	0.044	20	20 (100.0)	0	Analytical LoD
		HN	0.03	0.03	0.065	20	20 (100.0)	0	-
		CO	0.05	0.05	0.107	20	20 (100.0)	19 (95.0)	Cut-off
		LP	0.08	0.08	0.165	20	20 (100.0)	20 (100.0)	Above Cut-off
		MP	0.13	0.15	0.257	20	20 (100.0)	20 (100.0)	-
TBM119	ITD 24 bp	LN	0.01	0.02	0.023	20	20 (100.0)	0	Analytical LoD
		HN	0.02	0.03	0.047	20	20 (100.0)	0	-
		CO	0.04	0.05	0.089	20	20 (100.0)	20 (100.0)	Cut-off
		LP	0.07	0.08	0.152	20	19 (95.0)	19 (95.0)	Above Cut-off
		MP	0.13	0.13	0.269	20	20 (100.0)	20 (100.0)	-
TPB161	ITD 66 bp	LN	0.02	0.02	0.045	20	20 (100.0)	0	Analytical LoD
		HN	0.03	0.03	0.066	20	20 (100.0)	0	-
		CO	0.05	0.05	0.110	20	20 (100.0)	18 (90.0)	Cut-off
		LP	0.09	0.08	0.189	20	20 (100.0)	20 (100.0)	Above Cut-off
		MP	0.14	0.13	0.280	20	20 (100.0)	20 (100.0)	-
TPB329	ITD 217 bp	LN	0.01	0	0.073	20	2 (10.0)	0	Analytical LoD
		HN	0.02	0.02	0.147	20	15 (75.0)	0	-
		CO	0.04	0.04	0.276	20	20 (100.0)	9 (45.0)	Cut-off
		LP	0.08	0.08	0.539	20	19 (95.0)	19 (95.0)	Above Cut-off
		MP	0.13	0.13	0.838	20	20 (100.0)	20 (100.0)	-
True Neg ITD	None	TN	N/A	0	0	20	0	0	N/A
TrueNeg TKD	None	TN	N/A	0	0	20	0	0	N/A

*Classifications are defined as 1: Analytical LoD = lowest AR where samples were detected 95% of the time above the LoB, 2: cut-off is AR where samples were near SR 0.05, and 3: above Cut-off = lowest AR where samples could be detected 95% of the time above SR 0.05.

The AR does not represent the percent mutant in a specimen. The AR value was converted to % mutant. The LoD in terms of % mutation is shown in Table 9b below.

Table 9b. Analytical Sensitivity Allelic Ratio and % Mutant

Sample ID	Mutation	Mutation Classification	Above Cut-off 95% SR≥ 0.05		
			AR	SR	%Mut
FPB006	TKD I836	TKD I836 Deletion	0.144	0.07	12.6
TBM232	TKD D835	TKD D835 Substitution	0.089	0.05	8.2
TBM119	ITD 24bp	Small ITD Insert <30bp	0.107	0.05	9.7
TPB161	ITD 66bp	Medium ITD Insert 30-100bp	0.189	0.08	15.9
TPB329	ITD 217bp	Large ITD Insert ~200bp	0.539	0.08	35.0

3. Analytical Specificity

Cross-Reactivity

To assess the potential for amplification of non-specific products from human genomic DNA, *in silico* analysis of the PCR primers used in the assay was performed. No non-specific products were predicted for the assay.

Interfering Substances

Various endogenous and exogenous substances listed in Table 10 were evaluated for interference of the LeukoStrat® CDx FLT3 Mutation Assay. Thirty-seven clinical specimens were spiked with a mixture of exogenous and endogenous materials and compared to the same specimens spiked with only the solvents carrying the materials. The study consisted of 11 BM and 26 PB specimens with 15 FLT3 negative and 22 FLT3 positive specimens (14 ITDs and 8 TKDs).

Potential interfering substances were added to clinical specimens (both positive and negative for ITD and TKD mutations) and tested for mutation status based on mutant:wild type SR against the cutoff of 0.05 (negative <0.05, positive ≥0.05). Multiple substances (except wash buffer) were pooled and tested together due to limitations of sample volume and assay throughput. The substances were tested at the recommended testing levels per CLSI EP7-A2. No interference was observed based on mutation status using any of the potential interfering substances. The LeukoStrat®

CDx FLT3 Mutation Assay is capable of detecting ITD mutations sized 18 bp to 114 bp and TKD mutations in the presence the interferents shown in the table below.

Table 10. Potential Interfering Substances Tested

B (Blank)	I (Interferent)
• Water • Dimethylsulfoxide (DMSO) • Tris(hydroxymethyl)amino methane (Tris)	• 0.8 mg/mL sodium heparin in water • 60 mg/mL human serum albumin in water • 24 µg/mL cytarabine in water • 180 ng/mL daunorubicin in DMSO • 0.19 mg/mL bilirubin in Tris • 5% v/v Intralipid • 2 mg/mL hemoglobin

4. Reproducibility

The reproducibility and precision of the LeukoStrat® CDx FLT3 Mutation Assay was assessed in two studies, the first using cell line specimens and the second using clinical specimens.

a. Reagent lot and instrument variability

In the first study, each operator tested a nine-member test panel using three reagent lots and instruments. The capillary electrophoresis detection portion of the assay was performed on each of three instruments. All test steps utilizing different combinations of reagents and equipment were performed in parallel. Contrived samples comprised of blended cultured cell lines added to leukocyte depleted whole blood (from normal healthy donors) were tested as shown in Figure 2. The samples consisted of ITD samples containing inserts of 30 bp and 126 bp and the D835 TKD mutation. Samples represented low (near cutoff), mid, and high (100% mutant cell line) mutant:wild type SRs for small internal tandem duplication (ITD) insert, large ITD insert, and tyrosine kinase domain (TKD) mutation as shown in Figure 2. The study was conducted with 27 replicates per level. The acceptance criteria were that the total coefficient of variation (CV) shall be ≤25% for each panel member with a mean mutant:wild type SR ≥0.05.

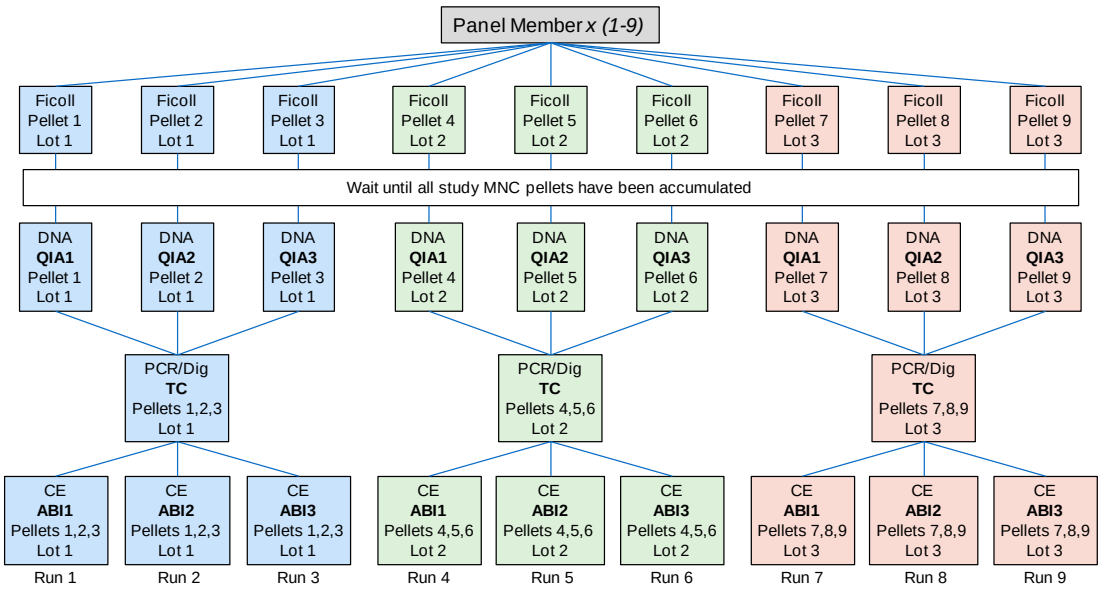


Figure 2. Workflow diagram, testing performed for each panel member by one operator. QIA=QIAcube; TC=thermal cycler; ABI=ABI 3500xL Genetic Analyzer. Extracted DNA from QIAcube instruments (3 replicates per reagent lot) was amplified together on 1 thermal cycler; each amplified plate was then tested on each of 3 CE instruments (to generate 9 mutant:wild type SRs per reagent lot).

The results showed that all mutant cells at each level tested positive (SR>0.05) and the percent coefficient of variation (%CV) was within the pre-specified acceptance criterion of 25% for this assay. For the ITD mutation samples, the SR %CV ranged from 3.0% to 8.4%. For the TKD mutation samples, the SR %CV ranged from 5.4% to 10.6%. Overall, the LeukoStrat® CDx FLT3 Mutation Assay lot-to-lot and instrument-to-instrument reproducibility has a SR %CV of less than 11% as summarized in Table 11.

Table 11. Components of Variance per Positive Panel Member, Mutant:Wild Type SR

PM	Description	Sample Size	SR Average	Variation due to			Total	Total CV, %
				Reagent Lot	Instrument	Random Error		
PM1	30 bp ITD, low	27	0.063	0.004 (61.67%)	0.001 (2.08%)	0.003 (36.25%)	0.005	7.4
PM2	30 bp ITD, mid,	27	0.918	0.000 (0.00%)	0.000 (0.00%)	0.045 (100.0%)	0.045	4.9
PM3	30 bp ITD, high	27	1.746	0.032 (33.77%)	0.000 (0.00%)	0.045 (66.23%)	0.055	3.0
PM4	126 bp, low	27	0.076	0.005 (58.60%)	0.001 (1.36%)	0.004 (40.05%)	0.007	8.4
PM5	126 bp, mid	27	0.144	0.008 (50.42%)	0.001 (1.12%)	0.008 (48.46%)	0.011	7.0
PM6	126 bp, high	27	0.379	0.008 (20.86%)	0.005 (7.41%)	0.014 (71.73%)	0.017	4.2

PM	Description	Sample Size	SR Average	Variation due to			Total	Total CV, %
				Reagent Lot	Instrument	Random Error		
PM7	D835, low	27	0.071	0.002 (25.00%)	0.001 (2.08%)	0.003 (72.92%)	0.004	5.4
PM8	D835, mid	27	0.157	0.004 (4.56%)	0.000 (0.00%)	0.016 (95.44%)	0.017	10.6
PM9	D835, high	27	0.309	0.000 (0.00%)	0.005 (4.83%)	0.021 (95.17%)	0.022	7.1

Variation is expressed in standard deviations. Values in parentheses are percentage of total variation for each source of variability. CV = coefficient of variation; PM = panel member.

b. Operator-to-operator and Between-run variability

Variability was evaluated for precision of the LeukoStrat® CDx FLT3 Mutation Assay. The same nine panel members tested in the lot-to-lot study were tested each by three different operators, using one reagent set and one instrument set, over 15 runs. Each of the operators processed a total of 10 replicates of a test panel member through mononuclear cells (MNC) collection (30 replicates total/panel member). For each panel member, MNCs were isolated over a one day processing period. Following accumulation of MNCs, samples were tested with the remainder of the assay over 31 calendar days. Acceptance criteria identified by the company are that the total CV shall be ≤25% for each panel member with a mean mutant:wild type SR ≥0.05.

The results showed that the percent coefficient of variation (%CV) for all ITD and TKD mutant cells at each level tested positive (SR>0.05) was within the acceptance criterion of 25%. For the ITD mutation samples, the SR %CV ranged from 6.6% to 13.3%. For the TKD mutation samples, the SR %CV ranged from 7.9% to 9.3%. Overall, the LeukoStrat® CDx FLT3 Mutation Assay lot-to-lot and instrument-to-instrument reproducibility has a SR %CV of less than 11% as summarized in Table 12.

Table 12. Components of Variance per Panel Member for Between-Operator and Between Run

PM	Description	Sample Size	SR Avg.	Variation due to			Total Variation	Total % CV
				Operator	Run Day	Random Error		
PM1	30 bp ITD, low	30	0.079	0.000 (0.31%)	0.005 (50.61%)	0.005 (49.08%)	0.007	9.4
PM2	30 bp ITD, mid	30	0.709	0.024 (16.70%)	0.028 (23.09%)	0.045 (60.21%)	0.058	8.2
PM3	30 bp, high	30	1.609	0.029 (7.38%)	0.092 (76.01%)	0.043 (16.61%)	0.106	6.6
PM4	126 bp, low	30	0.077	0.002 (4.39%)	0.007 (51.72%)	0.007 (43.89%)	0.010	13.3

PM	Description	Sample Size	SR Avg.	Variation due to			Total Variation	Total % CV
				Operator	Run Day	Random Error		
PM5	126 bp, mid	30	0.154	0.008 (19.19%)	0.013 (56.83%)	0.009 (23.98%)	0.018	11.6
PM6	126 bp	30	0.338	0.012 (16.52%)	0.025 (65.49%)	0.013 (17.99%)	0.030	9.0
PM7	D835, low	30	0.090	0.000 (0.00%)	0.005 (33.11%)	0.007 (66.89%)	0.008	9.3
PM8	D835, mid	30	0.156	0.000 (0.00%)	0.008 (42.67%)	0.009 (57.33%)	0.012	7.9
PM9	D835, high	28	0.304	0.012 (22.35%)	0.018 (48.48%)	0.014 (29.17%)	0.025	8.4

In the second study, reproducibility was assessed using clinical DNA samples from 8 clinical samples (4 PB and 4 BM) with ITD lengths of 21 bp, 24 bp, 66 bp, 90 bp and 217 bp, TKD D835 substitution, TKD I836 deletion, and FLT3 negative samples. DNA from FLT3 negative clinical specimens was pooled and used to dilute the FLT3 positive samples in order to achieve three target SR levels near the assay's clinical cut-off (i.e., high negative, low positive, and moderate positive). Five FLT3 positive clinical samples originated from PB and two from BM. Three replicates of 5 ITD positive, 2 TKD positive and one pooled true negative sample were tested by three different operators/instrument sets using 1 reagent lot over five non-consecutive days at three dilution levels for positive samples and neat for the negative. Each operator tested 15 replicates total per level for a total of 45 replicates per dilution level.

The total %CV of all mutation types and levels are shown in Table 13 and the %CV for all mutation types, except the long ITD insert (217 bp) sample, ranged from 4.2% to 16.1%. The sample with a 217 bp mutation %CV ranged from 26.9% to 27.2%. The low positive (LP) dilution level %CV was 26.9% for 217 bp, therefore failing the study acceptance criteria of ≤25% CV for SR. Results show that acceptance criteria were met for both D835 and I836 TKD mutations and for ITD mutations up to 217 bp. Variation for the 217 bp ITD mutation exceeded 25%, thus indicating greater imprecision around the largest ITDs.

Table 13. Components of Variance per Mutation Type and Dilution Level

Sample ID	Mut Type	Dilution Level	Mean SR	SR Variation Due to			Total Variation	
				Operator/ Instrument SD (%)	Run Day SD (%)	Random Error SD (%)	SD	% CV
FPB006	TKD I836	HN	0.03	0.000 (3.22%)	0.000 (0.00%)	0.002 (96.78%)	0.002	7.1
		LP	0.077	0.001 (2.60%)	0.000 (0.00%)	0.005 (97.40%)	0.005	5.9

Sample ID	Mut Type	Dilution Level	Mean SR	SR Variation Due to			Total Variation	
				Operator/ Instrument SD (%)	Run Day SD (%)	Random Error SD (%)	SD	% CV
		MP	0.132	0.002 (6.67%)	0.003 (17.43%)	0.005 (75.90%)	0.006	4.6
TPB262	TKD D835	HN	0.04	0.001 (7.13%)	0.000 (0.00%)	0.002 (92.87%)	0.002	5.3
		LP	0.08	0.002 (14.02%)	0.001 (2.47%)	0.004 (83.51%)	0.004	5.3
		MP	0.165	0.003 (16.28%)	0.000 (0.00%)	0.007 (83.72%)	0.007	4.2
TBM163	ITD 21 bp	HN	0.03	0.000 (0.00%)	0.000 (0.00%)	0.001 (100.0%)	0.001	5
		LP	0.074	0.000 (0.00%)	0.002 (8.08%)	0.005 (91.92%)	0.005	7.2
		MP	0.133	0.002 (14.5%)	0.000 (0.00%)	0.005 (85.54%)	0.006	4.4
TBM119	ITD 24 bp	HN	0.029	0.000 (0.00%)	0.000 (0.00%)	0.004 (100.0%)	0.004	15.2
		LP	0.07	0.000 (0.00%)	0.000 (0.92%)	0.004 (99.08%)	0.004	5.3
		MP	0.147	0.002 (8.20%)	0.001 (3.28%)	0.006 (88.52%)	0.007	4.5
TPB161	ITD 66 bp	HN	0.029	0.001 (4.28%)	0.000 (0.00%)	0.005 (95.72%)	0.005	16.1
		LP	0.083	0.000 (0.00%)	0.001 (1.13%)	0.007 (98.87%)	0.007	8
		MP	0.185	0.000 (0.00%)	0.000 (0.00%)	0.010 (100.0%)	0.01	5.3
TPB267	ITD 90 bp	HN	0.03	0.001 (5.15%)	0.000 (0.00%)	0.003 (94.85%)	0.003	10.1
		LP	0.091	0.004 (25.23%)	0.002 (8.42%)	0.007 (66.35%)	0.008	8.5
		MP	0.206	0.013 (44.26%)	0.005 (7.34%)	0.013 (48.40%)	0.019	8.5
TPB329	ITD 217 bp	HN	0.032	0.001 (0.90%)	0.002 (7.20%)	0.008 (91.90%)	0.009	27.2
		LP	0.079	0.013 (31.42%)	0.009 (14.86%)	0.017 (53.71%)	0.023	26.9
		MP	0.162	0.029 (36.75%)	0.015 (9.86%)	0.035 (53.39%)	0.047	27.2

Controls: Precision of the controls was also assessed and obtained from the above described studies. Results for the ITD and TKD positive controls for the lot-to-lot and operator-to-operator demonstrated that the between lot and between operator results were within 0.01 SR difference for ITD positive controls. For TKD positive control, the results were within 0.01 SR for between lot and .04 SR for between-operator. Data demonstrates that the precision on the controls was acceptable.

5. **Carryover (CO) and Cross-Contamination (CC)**

To assess for potential carryover and cross-contamination testing of the FLT3 Mutation Assay was performed over 4 ITD runs and 4 TKD runs. Samples were positioned throughout DNA extraction, amplification, digestion, and capillary electrophoresis plates to challenge carryover and cross-contamination conditions which could be expected to cause false positive results. No Template Control carryover testing was performed over 2 ITD runs and 2 TKD runs. On 96 well plates, alternating columns of positive amplicon and water were tested via capillary electrophoresis in order to capture carryover from positive samples into adjacent water filled wells.

For carryover and cross-contamination challenges, DNA was directly extracted from three cell lines (i.e., ITD positive, TKD positive, and wild type) over 7 QIAcube runs. Cell line positions on the QIAcube alternated between mutant positive and wild type. The isolated DNA was tested on 4 ITD and 4 TKD CE runs on the ABI3500xL instruments in the order of QIAcube extractions.

For NTC carryover challenge, water was carried through both the DNA amplification process and the EcoRV digestion. Columns of water samples were positioned adjacent to columns of positive amplicon samples in each of the 96-well plates and tested as 2 ITD runs and 2 TKD runs.

No carryover or cross-contamination was observed in the wild type (mutation negative) samples and no carryover was detected in the NTC (water) samples.

6. **Specimen Volume**

Two specimen volume studies using contrived samples or clinical specimens were carried out to determine the range of sample amounts necessary to perform the LeukoStrat® CDx FLT3 Mutation Assay.

a. **Specimen Volume 1 (Contrived Samples)**

The objective of this study was to demonstrate equivalency when using volumes of blood ranging from 0.9 to 3.3 mL in the FLT3 Mutation Assay. The assay is intended to use 3 mL PB specimen or 0.75 mL BM aspirate, when available. However, a minimum specimen volume of 1 mL PB and 0.25 mL BM aspirate allows testing of specimens with low volume.

Over 4 days, one operator processed 20 replicates of contrived test samples, consisting of FLT3-ITD mutant cells and FLT3 wild-type cultured cells, with the exceptions to sample and RPMI volumes listed below. There were 4 conditions each day, 5 replicates each. The DNA was extracted, and then frozen at -20˚C until the PCR step of the assay.

- Condition 1: 0.9 mL sample, 5.1 mL RPMI
- Condition 2: 1 mL sample, 5 mL RPMI
- Condition 3: 3.3 mL sample, 2.7 mL RPMI
- Condition 4: 1 mL sample, 3 mL RPMI

- Days 1 and 2: 5 replicates each of Conditions 1-4 on Low Challenge sample
- Days 3 and 4: 5 replicates each of Conditions 1-4 on High Challenge sample
- Low Challenge: 6 million cultured cells/ mL
- High Challenge: 100 million cultured cells/ mL

Mutant:Wild Type SR, DNA yield, and MNC counts were evaluated among the different specimen volumes. Means among the different sample volumes were compared using analysis of variance (ANOVA). For each sample, the mean values of SRs, DNA yield, and MNC concentration should not show a difference that is statistically significant, i.e., P-value should exceed 0.05 using ANOVA.

SRs were consistent across the 4 test conditions. This was the anticipated result when using different initial blood volumes over the Ficoll gradient, and the study supports that 3 mL PB can be processed over the Ficoll gradient. While there were some instances of statistically significant differences in mean SRs, the differences had no practical impact. The mean differences were small, and the clinical interpretation of the assay result would not change.

b. Specimen Volume 2 (Clinical Specimens)

The purpose of the study was to evaluate the variability, if any, in SR when processing different specimen volumes over the Ficoll gradient. Four clinical specimens, representing both ITD and TKD mutations and both specimen matrices were tested in triplicate under each of the following conditions:

- Condition 1: 0.9 mL PB and 5.1 mL RPMI; OR 0.22 mL BM and 5.78 mL RPMI
- Condition 2: 1 mL PB and 5 mL RPMI; OR 0.25 mL BM and 5.75 mL RPMI
- Condition 3: 3.3 mL PB and 2.7 mL RPMI; OR 0.83 mL BM and 5.17 mL RPMI

Specimens were tested at 90% minimum volume, nominal minimum volume, and 110% maximum volume in triplicate. Mutant:wild type SRs were compared and no changes in mean mutant:wild type SR were observed, demonstrating that LeukoStrat® CDx FLT3 Mutation Assay results were not affected by specimen volume and that a range of specimen volumes is acceptable for the assay.

7. Guard Band Studies

a. PCR Guard Band

The objective of this study was to evaluate the robustness of PCR to changes in temperature in the FLT3 Mutation Assay. The annealing, extension, and denaturation temperatures during PCR were tested at the nominal conditions shown in Table 14 and ±1°C FLT3-ITD positive control cell line, TKD positive control cell line, or wild-type cell line were tested using a total of 96 samples. Acceptance criteria were that for samples tested at nominal temperatures, control validity criteria were met. The results demonstrated the test was robust to temperature changes when detecting ITD mutations. However, the TKD was not

robust to changes in temperature. The instructions for use indicate that the PCR temperatures must be adhered to.

Table 14. Nominal PCR Conditions

Step	ITD Program	TKD Program
1	95°C for 11 minutes	95°C for 11 minutes
2	94°C for 30 seconds	94°C for 30 seconds
3	57°C for 60 seconds	57°C for 60 seconds
4	72°C for 2 minutes	72°C for 2 minutes
5	Repeat steps 2 to 4 24 times	Repeat steps 2 to 4 28 times
6	94°C for 30 seconds	94°C for 30 seconds
7	60°C for 45 minutes	60°C for 45 minutes
8	4°C forever	4°C forever

b. TKD Digestion
The objective of this study was to evaluate the robustness of the tyrosine kinase domain (TKD) mutation digestion step of the assay. Three samples representing a high TKD positive, a low TKD positive, and a TKD negative were tested for 1 hour (nominal condition) or 1.5 hours. Validity criteria were met.

c. Mononuclear Cell Centrifugation Temperature
The objective of this study was to evaluate the robustness of centrifuge temperature in the FLT3 Mutation Assay. Isolating MNCs from peripheral blood or bone marrow aspirate specimens uses centrifugation for the Ficoll gradient and wash steps. The centrifuge chamber temperature setting is 20°C. Two operators processed 16 contrived samples each. Each operator centrifuged half of their samples at 17°C and the other half at 23°C. Only the lower and upper bounds were tested as it was presumed that there was no non-linearity across the range of testing. The contrived samples were comprised of normal blood spiked with the ITD PC cells that have an ITD insertion of 30 bp. MNC counts, DNA yield, and mutant:wild type SR were measured; the values were evaluated to determine if they were equivalent between the two centrifuge temperature conditions. The mean values of MNC concentrations, DNA concentrations, and SR of the samples tested at the 17°C and the 23°C centrifuge temperatures should not show a difference that is statistically significant.

All samples (32/32 ITD, 100%) passed the ITD FLT3 validity criteria. The MNC concentrations, DNA concentrations, and SR from the two centrifuge temperatures were compared to each other. There was no significant difference observed under the tested conditions.

d. FLT3 Mutation Assay Guard Band: MNC Layer Collection
The objective of this study was to evaluate the robustness of cell collection in the assay. Mononuclear cells (MNC) were isolated from peripheral blood or bone marrow aspirate specimens using a Ficoll gradient. The MNC layer was manually

collected by aspiration with a transfer pipette. In the assay, the operator collected the MNC layer in up to 3 ml. Three operators collected 2.5, 3, and 3.5 ml of the MNC layer from contrived samples comprised of normal blood spiked with the 30 bp ITD PC. The SR, DNA Yield, and MNC Concentration mean values should not show a difference that is statistically significant.

Over a two day period, 63 contrived samples were made by 3 operators each processing 21 replicates of 1 ml. The replicates were divided among MNC layer collection volumes of 2.5, 3, and 3.5 ml (7 replicates each).

Table 15. Operator Variability of Final SR, DNA Yield, and MNC Concentration

	Operator	MNC Collection Volume (ml)	Mean	StDev	%CV	Max	Min	N
Final SR	1	2.5	0.033	0.008	23.5	0.049	0.025	7
		3	0.031	0.003	9.2	0.035	0.026	7
		3.5	0.033	0.006	18.3	0.044	0.025	7
	2	2.5	0.031	0.012	38.1	0.054	0.015	7
		3	0.024	0.005	19.0	0.034	0.020	7
		3.5	0.027	0.003	9.5	0.029	0.023	7
	4	2.5	0.058	0.009	15.2	0.050	0.066	7
		3	0.057	0.009	16.2	0.048	0.065	7
		3.5	0.055	0.005	9.6	0.051	0.060	7
DNA Yield (ng/µl)	1	2.5	27.5	3.8	13.7	31.2	22.0	7
		3	31.1	1.7	5.5	34.1	28.8	7
		3.5	29.1	5.2	18.0	34.0	18.7	7
	2	2.5	22.5	5.2	23.1	26.5	11.2	7
		3	27.4	4.6	17.0	33.7	20.1	7
		3.5	28.8	3.7	12.8	32.8	21.8	7
	4	2.5	54.1	8.62	15.9	46.2	62.1	7
		3	61.4	9.56	15.6	52.6	70.2	7
		3.5	68.6	3.41	5.0	65.5	71.8	7
MNC Conc. (x10^3 cells / µl)	1	2.5	0.14	0.05	37.4	0.2	0.1	7
		3	0.20	0	0	0.2	0.2	7
		3.5	0.16	0.05	34.0	0.2	0.1	7
	2	2.5	0.16	0.08	50.1	0.3	0.1	7
		3	0.17	0.05	28.5	0.2	0.1	7
		3.5	0.21	0.04	17.6	0.3	0.2	7
	4	2.5	0.23	0.049	21.3	0.3	0.2	7
		3	0.27	0.049	18.0	0.3	0.2	7
		3.5	0.34	0.053	15.6	0.4	0.3	7

Comparing the SR results across the 3 MNC layer collection volumes showed no significant difference in the 2.5, 3, or 3.5 ml MNC collection volumes (Table 15). The actual difference between the mean signal ratios of the 3 collection volumes was between 0 and 0.004 for samples. These differences in mean signal ratios are well within the signal ratio requirement for a %CV of 25%.

The DNA yield across the 3 MNC layer collection volumes was significantly different in both Batch 1 (operators 1 and 2) and 2 (operator 4) at a 5% level of confidence. In both batches, the 2.5 ml collection volume yielded a DNA concentration significantly lower than one of the other collection volumes. The difference in DNA yields across the 3 MNC layer collection volumes is expected since more cells are collected with the increasing volume. The DNA yields show a general increasing concentration with increasing MNC collection volume.

The MNC concentrations across the 3 MNC layer collection volumes showed no statistically significant difference in Batch 1 (p-value = 0.20) but show a statistically significant difference in Batch 2 (p-value = 0.007 < 0.05). However, like the DNA yield, it is expected that the MNC concentration would increase with the increased volume of MNC layer collected as seen in Batch 2.

Overall, the SRs showed no statistically significant differences between the 2.5, 3, and 3.5 ml collection volumes. Neither DNA yield nor MNC concentration had a significant impact on SR as proven by the similar SRs across the 3 MNC layer collection volumes.

e. DNA Input

The purpose of this study was to provide evidence that demonstrated equivalency when using DNA inputs at 10±3 ng/μL in the assay. Extracted DNA replicates from the Limit of Detection and Dynamic Range study with contrived samples were used by testing only the lowest allelic ratio sample panel members. DNA samples, listed below, were diluted to 7, 10, and 13 ng/μL and tested with the assay along with a single replicate of Negative Control.

- AR 0.03 30 bp ITD (33 replicates at each DNA input level)
- AR 0.05 D835 TKD (33 replicates)
- AR 0.05 126 bp ITD (22 replicates)
- AR 0.33 279 bp ITD (11 replicates)

Acceptance criteria were met for 30 bp ITD, 126 bp ITD, and D835 cells: 1) >93.9% of replicates met sample validity criteria for every sample type and DNA input; 2) overall coefficient of variation (CV) was <20.5% for every sample type; and 3) CV was <21.0% for every sample type when replicates were pooled between 7 and 10 ng/μL and between 13 and 10 ng/μL DNA input. Acceptance criteria were not met for TVI-ITD. While 100% of replicates met sample validity criteria, the overall CV and CV among pooled DNA inputs exceeded 25%.

The difference in mean mutant:wild type SRs among DNA inputs did not exceed 0.022, and the differences between means were not significantly different. The assay is able to provide consistent results when challenged with DNA inputs at 10±3 ng/μL.

f. Assay Stopping Points in the FLT3 Mutation Assay

Stopping points experienced during execution of the FLT3 Mutation Assay were evaluated in this study. DNA and amplified samples were incubated at the temperatures and durations sufficient to challenge typical work-flow stopping points. Samples studied were the same as from the precision/reproducibility study. Thus, only contrived samples were investigated. At least 25% extra time was added to the expected typical incubation times. Assay will allow three work flow stopping points:

1. After DNA isolation, samples can be held for up to 7 days at 2° to 8°C before proceeding
2. After PCR, samples can be held for up to 72 hours at 2° to 8°C before proceeding
3. After digestion step of TKD assay, samples can be held for up to 72 hours at 2° to 8°C before proceeding

The sample validity rate was required to be >95%. The qualitative test results were required to have at least 95% agreement with those obtained from the operator-to-operator precision study.

The following assay steps were performed: 1) samples were processed, mononuclear cells (MNCs) were isolated, and DNA wss extracted from the isolated MNCs (if time permits, DNA can be quantified); 2) DNA was quantified (if not already done) and diluted to 10 ng/μL, and the DNA wss amplified by PCR; 3) the tyrosine kinase domain (TKD) amplicons were digested with EcoRV restriction enzyme; and 4) the amplicons were analyzed by capillary electrophoresis (CE). The first step (sample processing through DNA extraction) must be completed in one day. While steps 2 to 4 above can be performed in one work shift, for workflow purposes pauses are allowed between steps 1 and 2, between steps 2 and 3, and between steps 3 and 4. All studies were conducted in duplicate.

<u>Stopping Point 1: DNA Extraction</u>

The DNA concentrations from the precision study were recorded and the samples were diluted to 10 ng/μl based on those measurements. The concentrated and diluted DNA samples were stored at 2° to 8° C for at least 9 days in duplicates for each of the 9 panel members. After 9 days, the concentrated DNA samples were diluted to10 ng/μl, and the assay (amplification through CE) was completed on all DNA samples.

Stopping Point 2: Amplification, and Holding Point: CE Preparation (ITD)
An amplified ITD PCR plate from a precision study run was obtained, and the plate was stored at 2° to 8°C for at least 90 hours. After the incubation period, the plate was prepared for CE analysis. The CE plate was stored on a 3500xL Genetic Analyzer for at least 15 hours and analyzed at the end of incubation.
This Stopping Point 2 is also referred to as the ITD Stopping Point, or ISP.

Stopping Points 2 and 3: Amplification and EcoRV Digestion, and Holding Point: CE Preparation (TKD)
An amplified TKD PCR plate from a precision study run was obtained, and the plate was stored at 2° to 8°C for at least 90 hours. After the incubation period, the plate was digested with EcoRV. The digested plate was stored at 2° to 8°C for at least 90 hours.
After the incubation period, the plate was prepared for CE analysis. The CE plate was stored on a 3500xL Genetic Analyzer for at least 15 hours and analyzed at the end of incubation. This Stopping Point, 2 and 3, is also referred to as the TKD Stopping Point, or TSP.

All Stopping Points and Holding Points
The ITD and TKD PCR plates from Stopping Point 1 were stored at 2° to 8°C for at least 90 hours. After the 90 hour incubation period, the ITD plate was prepared for CE analysis. The CE plate was stored on a 3500xL Genetic Analyzer for at least 15 hours and analyzed at the end of incubation. After the 90 hour incubation period, the TKD plate was digested with EcoRV. The digest plate was stored at 2° to 8°C for at least 90 hours. After the incubation period, the plate was prepared for CE analysis. The CE plate was stored on a 3500xL Genetic Analyzer for at least 15 hours and analyzed at the end of incubation.

The All Stopping Points and Holding Points are also referred to as the Cumulative Stopping Points, or CSP.
The qualitative test result (negative/positive) was determined for each sample based on the mutant:wild type signal ratio. A sample was called negative, i.e., mutation not detected because mutant:wild type signal ratio did not exceed the cutoff of 0.05, or positive, i.e., mutation detected because mutant:wild type signal ratio met or exceeded the cutoff of 0.05.

Results for each mutation type and stopping point are summarized in Table 21. All study acceptance criteria were met. Results show that correct calls can be made when incubation times are varied for different potential stopping points during the assay.

8. Stability Studies

a. Assay Kit Stability (shelf-life)
The purpose of this study was to provide objective evidence that the reagent stability requirements of the LeukoStrat® CDx FLT3 Mutation Assay would be

met. Each kit lot was functionally tested at time points 0, 2, 3 and 4 months using 2 replicates of the 30 bp ITD and D835 TKD DNA positive control, no template control, extraction control or cell based positive controls in the ITD and TKD portions of the assay. Acceptance criteria were that kits had to yield $\geq$9.5 ng/µl upon extraction and meet validity criteria. The study demonstrates that kits are stable for at least 3 months from the date of manufacture when components are stored at the recommended temperatures (-30°C to -15 °C, 2 °C to 8 °C, or 15 °C to 30 °C.

b. Open Kit Stability

To provide proof that all reagents are stable for at least one week after being opened, used and stored per the LeukoStrat® CDx FLT3 Mutation Assay instructions, two ITD Positive Cell Based Controls (ITD CBC---30 bp ITD) and two TKD Positive Cell Based Controls (TKD CBC) were tested in the FLT3 Mutation Assay twice over the span of a week. Three kit lots were tested on Day 1 and Day 8 using kits that were 4 months old. ITD and CBC SR had to be >0.05 in the ITD reaction and <0.05 for the TKD reaction. TKD CBC SR had to be >0.05 in the TKD reaction and <0.05 in the ITD reaction.

The ITD CBC SRs and TKD CBC SRs were independent of the time point, Day 1 or Day 8, and size standard solution, verifying that all reagents are stable when opened and used multiple times over eight days.

c. Capillary Stability

The purpose of this study was to provide objective evidence that the stability requirements of the ABI3500xL Capillary Array would be met. Capillaries' abilities to appropriately separate products were evaluated using sizing accuracy of Map Marker and DS-33 GeneScan Installation Standard fragments; signal sensitivity was evaluated using DS-33 GeneScan Installation Standard and Size Standard fragments. Each capillary array lot was functionally tested at time points 0, 300, 400, and 500 injections to demonstrate performance of the capillary arrays are stable for a maximum of 400 injections.

The report provides objective evidence that three capillary array lots have successfully completed all 4 times points, indicating they are stable for at least 400 injections.

d. Reagent Stability

i. Master Mixes and Controls

The purpose of this study was to provide evidence that the reagent stability requirements of the LeukoStrat® CDx FLT3 Mutation Assay were met. Invivoscribe manufactured reagents must be stable for at least 6 months when stored under recommended conditions. Three kit lots were tested with each of the assay controls [positive control (PC), extraction control (EC), no template control (NTC)] at 0, 6, and 8 months. The 6 month time point showed 100% agreement with the baseline values for the 54 replicates, for each control (162

total replicates) while the 8 month time point had 53/54 (98%) PC replicates and 54/54 replicates of both EC and NTC. Each time point was comprised of 6 runs, 3 ITD and 3 TKD. Each lot of Master Mix was tested with all 3 lots of Controls – 3 vials per Control, 2 replicates per vial. Testing was divided among three operators. The 8 month time point had 53/54 (98%) PC replicates and 54/54 replicates of both EC and NTC meeting the predefined material acceptance criteria of 99%for all controls).

ii. EcoRV, Buffer 3 and BSA Stability

The performance of EcoRV Enzyme, Buffer 3 and 100 µg/mL BSA was evaluated at three time points of 0 months, 6 months, and 8 months. Each reagent was tested together as a set at each time point. Each set consisted of 3 vials with three replicates of each vial that were averaged together to produce an Average Enzyme Activity for the vial. All replicates were above the specification of Average Enzyme Activity per triplicate ≥ 2.55 Units/mL..

This study provides evidence that the EcoRV Enzyme, Buffer 3 and 100 µg/mL of BSA have a shelf life of six months.

iii. Formamide & Dye size standard Stability

Amplicons generated using the FLT3 Mutation Assay must be denatured into single stranded products and sized using ABI3500xL instrument. Highly deionized (HIDI) formamide is used to keep the products single stranded. Dye size standard allows the products to be accurately sized by the instrument. Because HIDI formamide's and size standard's stability has a direct impact on the FLT3 Mutation Assay's performance, this stability study was intended to support HIDI formamide's and size standard's stability. This study was carried out using three lots of HIDI formamide and three lots of size standard with only the TKD injections mixed with Map Marker and DS-33 GeneScan Installation Standard for denaturation.

For the time point to be considered acceptable, 3 lot pairs of HIDI formamide and size standard had to meet the validity and acceptance criteria for the expected size fragment and a minimum peak size of 100 RFU for the 20 bp fragment or >250 RFU for all other fragments for each time point (0, 6, and 8 months). This study provided evidence that HIDI formamide and size standards are stable up to at least 6 months by yielding the correct fragment sizes and peak heights.

iv. Taq Polymerase Stability

The purpose of this study was to provide objective evidence that the LeukoStrat® CDx FLT3 LeukoStrat® CDx FLT3 Mutation Assay reagents: Taq Polymerase, Master Mix, Positive Control, Extraction Control and No Template Control are stable for at least 6 months when stored under recommended conditions.

Three combinations were constructed and tested at each time point:
- Combination 1: MM1-T1-PC1-EC1-NTC1
- Combination 2: MM2-T2-PC2-EC2-NTC2
- Combination 3: MM3-T3-PC3-EC3-NTC3

For each combination, 6 replicates of each Control were made from a mix of each Master Mix/Taq lot.

Each lot of controls tested has met stability requirements for the LeukoStrat® CDx FLT3 LeukoStrat® CDx FLT3 Mutation Assay over 8 months. Therefore, reagents manufactured at Invivoscribe were stable for 6 months when stored under recommended conditions. The baseline had 52/54 (96%) ITD and 54/54 (100%) TKD replicates that met the acceptance criteria. The 6 and 8 month time points had 100% (54/54) of all control ITD and TKD replicates that met the acceptance criteria.

All time points tested to date following 0 months/baseline, were completed within the 14 day window allowed by the protocol. This study covers time points 0 (baseline), 6, and 8 months.

As of the 8 month time point, the study meets the acceptance criteria of 6 months of stability according to assay requirements with only two invalid results across all time points.

When stored under recommended conditions, the combination of LeukoStrat® CDx FLT3 Mutation Assay reagents (Taq Polymerase, Master Mix, Positive Control, Extraction Control and No Template Control) is stable for 6 months.

e. Specimen Stability

The objective of this study was to evaluate the stability of BM aspirate and PB specimens collected from patients with acute myeloid leukemia (AML). This study presents the FLT3 Mutation Assay results of 52 BM and 100 PB clinical specimens. Specimens were processed to DNA upon receipt (up to 4 days after collection) then stored at 2° to 8° C and processed again at Day 5 and Day 7 after collection. If volume permitted, specimens were processed again on Day 9. DNA isolated from the specimens was stored at -15° to -30° C and tested up to 35 months later once design lock had been achieved and all samples were accrued.

For BM, NPA from Baseline to Day 7 for FLT3 mutation (ITD and TKD mutations combined) was 100% (21/21), and PPA was 100% (30/30). NPA was required to be at least 95% and PPA was required to be at least 90%; therefore, the acceptance criteria for BM were met for this study. For PB, NPA from Baseline to Day 7 for FLT3 mutation (ITD and TKD mutations combined) was 97.9% (47/48), and PPA was 98.0% (50/51). NPA was required to be at least 95% and PPA was required to be at least 90%; therefore, the acceptance criteria for PB were

met for this study. The Baseline to Day 9 FLT3 NPA and PPA for BM was 100% (17/17) and 100% (24/24), respectively. The baseline to Day 9 FLT3 NPA and PPA for PB was 96.6% (28/29) and 100% (43/43), respectively.

This study supports the claim that specimens will be analyzable up to 5 days following date of collection if refrigerated at between 2°C to 8°C.

B. Animal Studies

Not Applicable

X. SUMMARY OF PRIMARY CLINICAL STUDY(IES)

The safety and effectiveness of the LeukoStrat® CDx FLT3 Mutation Assay were demonstrated in a retrospective analysis of specimens from patients screened for enrollment into the RATIFY clinical trial. The RATIFY (*Randomized AML Trial In FLT3 in <60 Year Olds*, also known as CPKC412A2301 (or A2301) clinical trial was a Phase 3, randomized, double-blind placebo-controlled trial of 717 patients with newly-diagnosed FLT3-mutated AML to assess the safety and efficacy of RYDAPT (midostaurin) in combination with chemotherapy. A bridging study was conducted to assess clinical agreement between samples with FLT3 status tested with the clinical trial assay (CTA) and the LeukoStrat® CDx FLT3 Mutation Assay in the intent-to-test population. The primary objectives of the study were to 1) establish agreement with respect to selection of FLT3 mutant patients between the CTA and the LeukoStrat® CDx *FLT3* Mutation Assay by assessing the overall, positive, and negative percent agreement between the two assays and 2) to estimate midostaurin efficacy in the LeukoStrat® CDx *FLT3* Mutation Assay positive population on both overall survival (OS)

A. Study Design

The RATIFY clinical trial was a multi-site, international, Phase 3, randomized, double-blind trial assessing the use of midostaurin in combination with standard remission induction and consolidation chemotherapy (n = 345), compared to placebo with chemotherapy (n = 335) for treatment of patients with newly-diagnosed FLT3-mutant AML. The original trial design allowed for initial screening for FLT3 mutation in either peripheral blood (PB) or bone marrow (BM) sample of patients with newly-diagnosed AML. Patients were stratified at randomization based on FLT3-ITD allelic ratio < 0.7, FLT3-ITD allelic ratio ≥ 0.7, and FLT3-TKD mutations. The primary endpoint was overall survival.

1. Clinical Inclusion and Exclusion Criteria
   Enrollment in the RATIFYstudy was limited to patients who met the following inclusion criteria
   - Unequivocal diagnosis of AML (>20% blasts in the bone marrow) excluding acute promyelocytic leukemia without evidence of central nervous system involvement.

- Documented FLT3 mutation (ITD or TKD), determined by analysis in a protocol-designated FLT3 screening laboratory.
- Age of subject ≥18 and <60 years.

Patients were not permitted to enroll in the RATIFY study if they met any of the following exclusion criteria:

- Prior chemotherapy for leukemia or myelodysplasia with the following exceptions:
  - emergency leukapheresis
  - emergency treatment for hyperleukocytosis with hydroxyurea for ≤ 5 days,
  - cranial radiation therapy for CNS leukostasis (one dose only),
  - growth factor/cytokine support.
- Patients with a history of antecedent myelodysplasia (MDS) who had prior cytotoxic therapy (including azacitidine or decitabine) for MDS.
- Patients who have developed therapy-related AML after prior RT or chemotherapy for another disorder or cancer
- Patients with symptomatic congestive heart failure.
- Bilirubin ≥2.5 x upper limit of normal.
- Pregnant or nursing.

Additional Specimen Inclusion/Exclusion Criteria for the Bridging Study
Inclusion Criteria

- DNA isolated from CTA(+) and CTA(-) subjects screened for the RATIFY trial.
- DNA isolated from MNCs frozen in a method shown to adequately preserve the specimen.
- DNA of a quantity of at least 50ng at ≥10ng/μL (defined as ≥9.5ng/μL in the assay IFU).

Exclusion Criteria

- Sample delivered without legible unique identification number.
- Obvious physical damage to sample tube.
- DNA received thawed.

2. Follow-up Schedule
   Disease assessment and other clinical assessments were conducted according to schedule. The primary analysis was conducted after a minimum follow-up of approximately 3.5 years after the randomization of the last patient. Efficacy was also assessed by event-free survival (EFS) where an event was defined as a failure to obtain a complete remission (CR) within 60 days of initiation of protocol therapy, or relapse, or death from any cause.

3. Clinical Endpoint
   The primary objective of the A2301 study was to determine if the addition of midostaurin to daunorubicin/cytarabine induction, high-dose cytarabine

consolidation, and continuation therapy improved OS in both mutant FLT3-ITD or FLT3-TKD AML patients.

B. Accountability of PMA Cohort

A total of 3279 patients were screened for the presence of FLT3 mutations by the clinical trial assay (CTA) and enrollment criteria. Of those, 717 patients with FLT3 mutations were enrolled and randomized to receive treatment with chemotherapy plus midostaurin or placebo. Only patients with FLT3 mutations and meeting all trial inclusion criteria were enrolled. The bridging study included the ascertained samples from the RATIFY (A2301) patients who had provided informed consent for LeukStrat CDx FLT3 Mutation Assay (CDx) development. These samples were from the CTA+ patients enrolled in the A2301 study as well as the non-enrolled screen failures. Banked A2301 samples from 520 CTA-positive patients and 578 randomly selected CTA-negative patients were tested by the CDx test. The CTA and the CDx results were used to evaluate the concordance (agreement) of the assays as well as to estimate the midostaurin efficacy in the CDx-positive population. Reasons for unavailability of specimens are shown in Figure 3 below. (Figure 3 also refers to selection of specimens for the method comparison (accuracy/correlation to another method study described above).

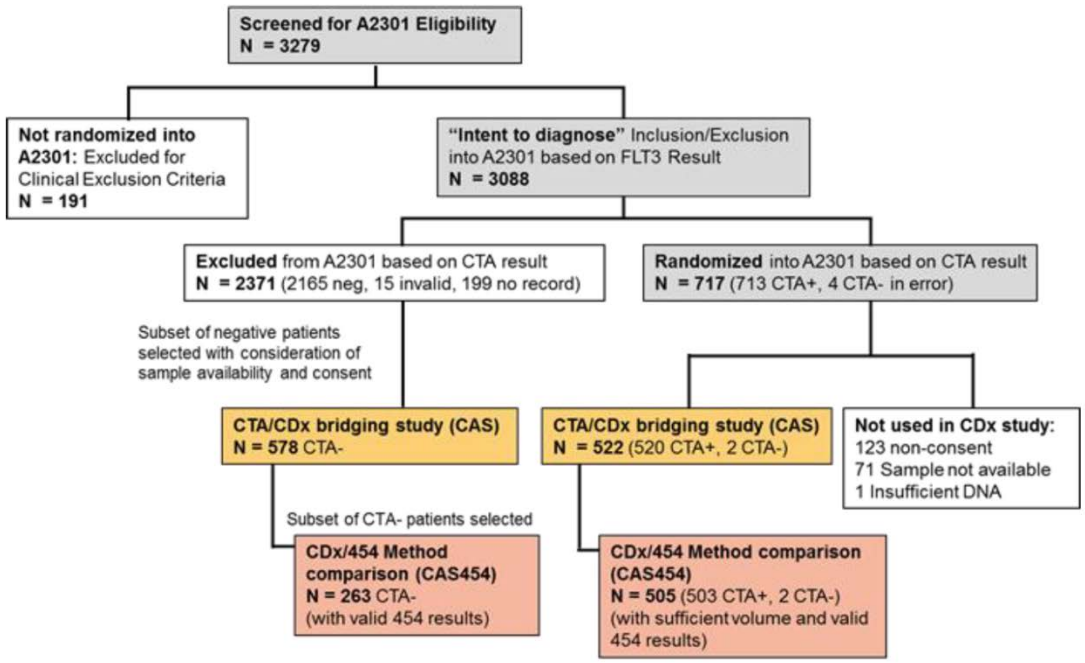


Figure 3. AML Specimen Accountability for CDx Bridging Study.
For the bridging study, 578 CTA- patients were selected but only 555 CTA- specimens were evaluable by CDx analysis testing due to low sample. For the CTA+ specimens, 498 were evaluable by CDx analysis.

C. Study Population Demographics and Baseline Parameters

In the RATIFY trial, 3279 patients were screened to enroll 717 who were positive for FLT3 mutations based on the CTA test. The two treatment groups were generally balanced with respect to the baseline demographics (Tables 23). The median age of study participants was 47 years on the midostaurin arm and 48 years on the placebo arm, with a range of 18-59 years. Race was unknown or not reported in 57% of subjects, but 89% of the subjects for whom race was reported were white. Distribution of FLT3 based on AR stratification factor was the same across both arms. The distribution of several characteristics for those concordant cases used in the bridging study and by enrollment site and testing site is shown in the Table 16.

Table 16. Comparison of baseline characteristics and sample covariates between the two treatment arms in the (CTA+, CDx+) patients

Baseline Characteristics (CTA+)	MIDOSTAURIN N=252	PLACEBO N=237	ALL N=489	p-value (1)
Region - n (%)				0.999
North America	101 (40.1)	95 (40.1)	196 (40.1)	
Non North America	151 (59.9)	142 (59.9)	293 (59.9)	
Country - n				0.964
Austria	4 (1.6)	3 (1.3)	7 (1.4)	
Belgium	3 (1.2)	4 (1.7)	7 (1.4)	
Canada	2 (0.8)	5 (2.1)	7 (1.4)	
Czech Republic	2 (0.8)	3 (1.3)	5 (1.0)	
France	3 (1.2)	2 (0.8)	5 (1.0)	
Germany	112 (44.4)	105 (44.3)	217 (44.4)	
Italy	18 (7.1)	15 (6.3)	33 (6.7)	
Netherlands	3 (1.2)	2 (0.8)	5 (1.0)	
Slovakia	2 (0.8)	1 (0.4)	3 (0.6)	
Spain	4 (1.6)	7 (3.0)	11 (2.2)	
United States	99 (39.3)	90 (38.0)	189 (38.7)	
CTA Laboratory				0.957
OS	101 (40.1)	95 (40.1)	196 (40.1)	
HA	13 (5.2)	12 (5.1)	25 (5.1)	
UL	69 (27.4)	71 (30.0)	140 (28.6)	
DR	36 (14.3)	28 (11.8)	64 (13.1)	
UR	18 (7.1)	15 (6.3)	33 (6.7)	
NI	11 (4.4)	9 (3.8)	20 (4.1)	
BA	2 (0.8)	3 (1.3)	5 (1.0)	
VA	2 (0.8)	4 (1.7)	6 (1.2)	
Sample Material Banked by the laboratory				0.751
DNA	11 (4.4)	9 (3.8)	20 (4.1)	
MNC	241 (95.6)	228 (96.2)	469 (95.9)	
MNC count is greater than 5x10^5 cells				0.929
Yes	57 (22.6)	52 (21.9)	109 (22.3)	
No	184 (73.0)	176 (74.3)	360 (73.6)	
Missing	11 (4.4)	9 (3.8)	20 (4.1)	
FLT3 mutation status - n (%)(3)				0.539
TKD	59 (23.4)	50 (21.1)	109 (22.3)	
ITD (includes patients with both TKD and ITD)	193 (76.6)	187 (78.9)	380 (77.7)	
ITD Signal ratio <0.7	111 (44.0)	112 (47.3)	223 (45.6)	
ITD Signal ratio >=0.7	82 (32.5)	75 (31.6)	157 (32.1)	

D. Safety and Effectiveness Results

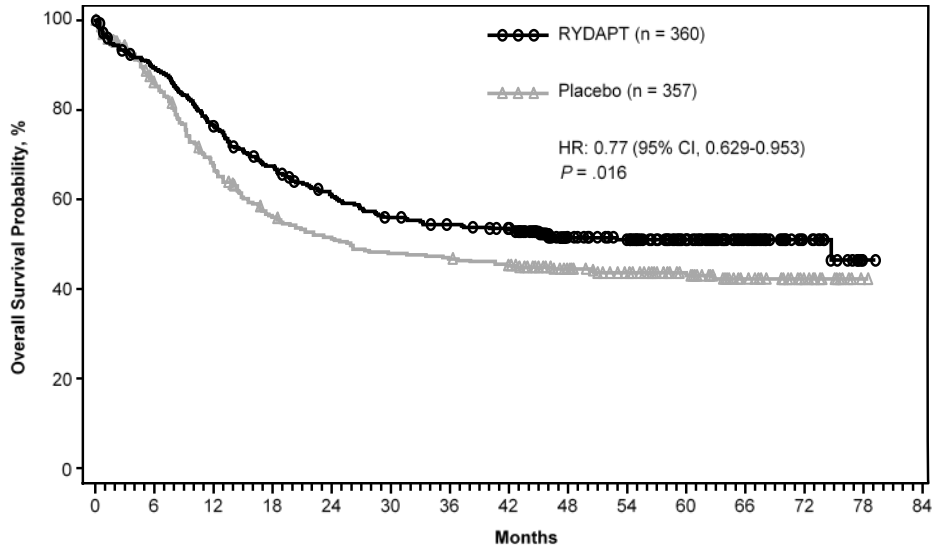
1. Safety Results

The safety with respect to treatment with RYDAPT (midostaurin) will not be addressed in detail in the SSED. Briefly, the most frequent (incidence greater than or equal to 20%) adverse drug reactions (ADRs) in the RYDAPT plus chemotherapy arm were febrile neutropenia, nausea, vomiting, mucositis, headache, musculoskeletal pain, petechiae, device-related infection, epistaxis, hyperglycemia and upper respiratory tract infections. Discontinuation due to any adverse reaction occurred in 9% of patients in the RYDAPT arm versus 6% in the placebo arm. The most frequent Grade 3/4 adverse reactions leading to discontinuation in the RYDAPT arm was renal insufficiency (1%). Excluding deaths due to disease progression, no fatal adverse reactions occurred in the study. Refer to the drug label for additional information.

The LeukoStrat™ CDx FLT3 Mutation Assay involves testing on PB and BM specimens. These specimens are routinely removed as part of the practice of medicine for the diagnosis of AML and therefore present no additional safety hazard to the patient being tested. See the Safety Conclusions Section below for further discussion.

2. Effectiveness Results
   a. Efficacy based on CTA:
      Efficacy was established on the basis of overall survival (OS), measured from the date of randomization until death by any cause. The primary analysis was conducted after a minimum follow-up of approximately 3.5 years after the randomization of the last patient. RYDAPT plus standard chemotherapy was superior to placebo plus standard chemotherapy in OS (HR 0.77; 95% CI 0.63, 0.95; 2 sided p=0.016) (Figure 1). Because survival curves plateaued before reaching the median, median survival could not be reliably estimated.

Figure 4: Kaplan-Meier Curve for Overall Survival in the Study



Patients at risk

Months	0	6	12	18	24	30	36	42	48	54	60	66	72	78	84
Midostaurin	360	314	269	234	208	189	181	174	133	120	77	50	22	1	0
Placebo	357	284	221	179	163	152	148	141	110	95	71	45	20	1	0

b. Bridging Study

i. Agreement between CTA and LeukoStrat® CDx FLT3 Mutation Assay – Overall

To support the safety and efficacy assessment of the LeukoStrat® CDx FLT3 Mutation Assay, clinical agreement was required to be demonstrated between samples with FLT3 status determined from the CTA and the LeukoStrat® CDx FLT3 Mutation Assay in the intent-to-test population. Overall agreement between the CTA and the CDx results was calculated based on FLT3 mutation status, which included both the evaluation of ITD and TKD mutations. The sample set was comprised of 503 CTA-positive and 555 CTA negative samples (Tables 18 and 19). Among the 503 CTA-positive samples, 489 returned a positive result with the CDx. Among the 555 CTA-negative samples, 540 returned a negative result with the CDx. Twelve patients failed to yield valid results with the CDx. There were 17 discordant results among 1058 samples analyzed. Of the 17 discordant results, 15 were associated with samples near the clinical cutoff (SR $\geq$ 0.05). For the two remaining discordants, high throughput sequencing confirmed the CDx results.

Table 18. Concordance table between CDx and CTA

FLT3 CDx	FLT3 CTA	
	Positive	**Negative**
Positive	489	8
Negative	9	540
Invalid	5	7
Total	503	555
- Invalid means that a sample was tested on the CDx assay but failed to return a valid result. - The CDx results from low DNA samples were excluded from this analysis.		

Table 19. Agreement between CDx and CTA based on CTA results

Measure of Agreement	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	**95% CI (1)**	**Percent Agreement (N)**	**95% CI (1)**
PPA	98.2% (489/498)	(96.6%, 99.2%)	97.2% (489/503)	(95.4%, 98.5%)
NPA	98.5% (540/548)	(97.1%, 99.4%)	97.3% (540/555)	(95.6%, 98.5%)
OPA	98.4% (1029/1046)	(97.4%, 99.1%)	97.3% (1029/1058)	(96.1%, 98.2%)
(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method - Invalid means that a sample was tested on the CDx assay but failed to return a valid result. - The CDx results from low DNA samples were excluded from this analysis.				

In the original clinical study, the prevalence of CTA-positive (n=713) was 24.8% among the screened patients who would otherwise meet the clinical inclusion criteria (n=2874). The agreements between CDx and CTA adjusting for the enrichment are shown in Table 20.

Table 20. Agreement between CDx and CTA based on CDx results, adjusted for enrichment

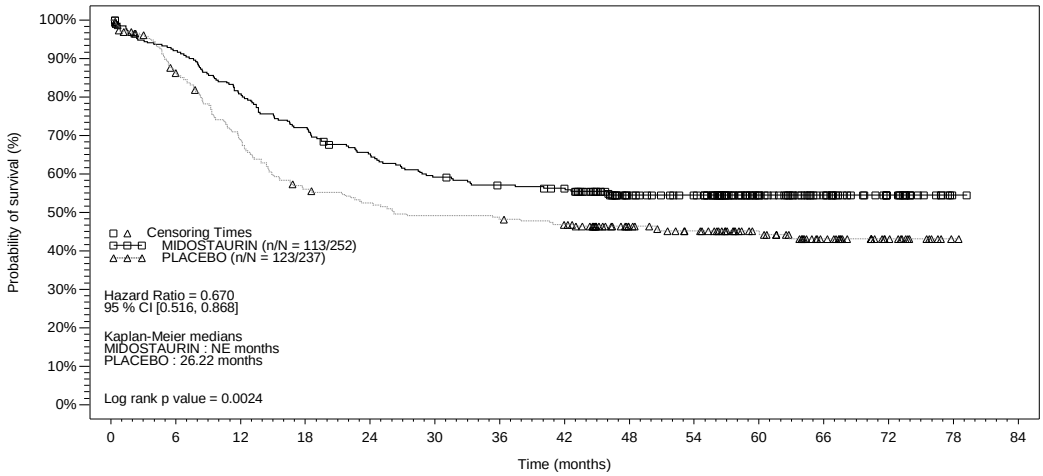
Measure of Agreement	Percent Agreement	95% CI(1)
PPA	95.7%	(92.8%, 98.3%)
NPA	99.4%	(99.0%, 99.7%)
OPA	98.5%	(97.6%, 99.2%)
(1) The 95% CI was calculated using non-parametric bootstrapping method.		

ii. Effectiveness in the (CTA+, CDx+) population (489 subjects):

Midostaurin efficacy on overall survival in the CDx-positive population was estimated. Efficacy determined in the (CTA+, CDX+) population was similar between the CDx tested subset and the overall A2301 clinical trial results for both overall survival with non-censoring at stem cell transplant.

Hazard ratio (95% CI) outcomes for OS were 0.67 (0.52, 0.87) vs A2301 OS 0.77 (0.63, 0.95) as shown in Figure 5

Figure 2 Kaplan-Meier for overall survival in the A2301 Trial in the CTA+, CDx+ Population.



No. of patients still at risk

Time (months)	0	6	12	18	24	30	36	42	48	54	60	66	72	78	84
MIDOSTAURIN	252	230	202	180	161	146	139	135	103	93	58	38	19	1	0
PLACEBO	237	193	152	123	114	107	105	100	77	67	50	31	14	1	0

The CDx results from low quantity samples are excluded from this analysis.
Logrank test and Cox regression model are stratified by the FLT3 CDx mutation strata. Hazard ratio and 95% CI are from the adjusted model.

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iii. Effectiveness in the (CDx+) population

The LeukoStrat® CDx FLT3 Mutation Assay population included two sub-populations in the clinical trial. One population defined as CDx+/CTA+ had clinical outcome (see Section D.2.b.ii. above). The other population defined as CDx+/CTA- had no clinical outcome because the LeukoStrat® CDx FLT3 Mutation Assay was not used to select those patients for the clinical trial. Thus, additional efficacy analyses were conducted to consider patients who were not included in the clinical trial because they were tested negative by the CTA, but could have been tested positive by the CDx (i.e., CDx+/CTA-).

Sensitivity analysis was conducted for both not censoring and censoring at the time of SCT using the analysis models adjusted for treatment and the 8 clinically relevant covariates which include age, gender, race, FAB class, WHO diagnosis criteria, PB blast %, BM blast %, and CTA laboratory. The hazard ratio estimates ranged from 0.63 with two-sided 95% CI of (0.48, 0.82) to 0.64 (0.49,0.84) for not censoring at the time of SCT and 0.51 (0.33, 0.78) to 0.52 (0.34, 0.81) for censoring at the time of SCT and

are similar to the results for the (CTA+, CDx+) population. When adjusted for treatment and gender, the hazard ratio estimates for overall survival ranged from 0.69 (0.54, 0.89) to 0.71 (0.55, 0.90) for not censoring and 0.68 (0.46, 0.99) to 0.69 (0.47, 1.01) for censoring at the time of SCT and are similar to the results for the (CTA+, CDx+) population.

Two additional imputation models were used to impute the 22 missing CDx results (CDx unevaluable) in the FAS-RATIFY using the same 8 clinically relevant covariates. The results of these analyses support the evidence that conclusions from the bridging study are representative of results obtained in the trial with the CTA.

3. Subgroup Analyses

a. Agreements between CTA and CDx by mutation types:

Table 22 and Table 23 show the concordance and agreement between the CDx ITD assay and the CTA ITD assay using the CTA ITD assay as reference.

Table 22. Concordance table between ITD CDx and ITD CTA, excluding the CDx results from low quantity samples (CAS)

	ITD CTA	
ITD CDx	**+**	**-**
+	378	5
-	6	660
Invalid	4	5
Total	388	670

- Invalid means that a sample was tested on the CDx assay but failed to return a valid result.
- The CDx results from low quantity samples are excluded from this analysis.

Table 23. Agreement on ITD between CDx and CTA based on CTA results

Measure of Agreement	**Without CDx Invalid**		**With CDx Invalid**	
	Percent Agreement (N)	**95% CI(1)**	**Percent Agreement (N)**	**95% CI(1)**
PPA	98.4% (378/384)	(96.6%, 99.4%)	97.4% (378/388)	(95.3%, 98.8%)
NPA	99.2% (660/665)	(98.3%, 99.8%)	98.5% (660/670)	(97.3%, 99.3%)
OPA	99.0% (1038/1049)	(98.1%, 99.5%)	98.1% (1038/1058)	(97.1%, 98.8%)

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method
- Invalid means that a sample was tested on the CDx assay but failed to return a valid result.
- The CDx results from low quantity samples were excluded from this analysis.

Table 24 and Table 25 show the concordance and agreement between the CDx TKD assay and the CTA TKD assay using the CTA TKD assay as reference. The sample set was comprised of 135 TKD positive and 922 TKD negative samples.

Table 24. Concordance table on TKD between CDx and CTA

	TKD CTA	
TKD CDx	+	-
+	127	12
-	6	902
Invalid	2	8
Total	135	922

- Invalid means that a sample was tested on the CDx assay but failed to return a valid result.
- The CDx results from low quantity samples are excluded from this analysis.

Table 25. Agreement on TKD between CDx and CTA based on CTA results (CAS)

Measure of Agreement	**Without CDx Invalid**		**With CDx Invalid**	
	Percent Agreement (N)	**95% CI [(1)]**	**Percent Agreement (N)**	**95% CI [(1)]**
PPA	95.5% (127/133)	(90.4%, 98.3%)	94.1% (127/135)	(88.7%, 97.4%)
NPA	98.7% (902/914)	(97.7%, 99.3%)	97.8% (902/922)	(96.7%, 98.7%)
OPA	98.3% (1029/1047)	(97.3%, 99.0%)	97.4% (1029/1057)	(96.2%, 98.2%)

(1) The 95% CI was calculated using the Exact (Clopper-Pearson) method
- Invalid means that a sample was tested on the CDx assay but failed to return a valid result.
- The CDx results from low DNA samples were excluded from this analysis.

b. Effectiveness in the subgroups: (ITD+) and (TKD+) population

The subgroup analysis by mutation type was performed in the A2301 population. The HR (95% CI) for OS (non-censoring at the time of stem cell transplantation) was estimated to be 0.65 (0.39, 1.09) in the TKD group and 0.80 (0.64, 1.01) in the ITD group, while the HR (95% CI) for OS (censoring at the time of stem cell transplantation) was estimated to be 0.90 (0.44, 1.85) in the TKD group and 0.70 (0.49, 1.00) in the ITD group.

Subgroup analysis by mutation type was also performed in the (CTA+, CDx+) population using the strata defined by the CDx results. For OS non-censoring at the time of stem cell transplantation, the HR (95% CI) was estimated to be 0.64 (0.35, 1.19) in the TKD group and 0.71 (0.54, 0.94) in the ITD group.

4. Pediatric Extrapolation

In this premarket application, existing clinical data was not leveraged to support approval of a pediatric patient population.

E. Financial Disclosure

The Financial Disclosure by Clinical Investigators regulation (21 CFR 54) requires applicants who submit a marketing application to include certain information concerning the compensation to, and financial interests and arrangement of, any clinical investigator conducting clinical studies covered by the regulation. The pivotal clinical study included 1investigator. None of the clinical investigators had disclosable financial interests/arrangements as defined in sections 54.2(a), (b), (c), and (f). The information provided does not raise any questions about the reliability of the data.

XI. SUMMARY OF SUPPLEMENTAL CLINICAL INFORMATION

To evaluate whether genomic DNA (gDNA) isolated from either BM aspirates or PB yielded concordant results, paired samples of PB and BM aspirates from the same patient were tested by the CDx test. The PB and BM analysis includes a subset of 184 patients, including 93 FLT3 mutation positive and 91 FLT3 negative specimens, in the CAS who have valid CDx results from both PB and BM samples. Analyses were conducted for overall mutation concordance as well as by ITD and TKD mutation agreement. Acceptance criteria was set at 90% for the lower bounds of the 95% confidence intervals for average positive and average negative agreement.

FLT3 mutations were detected in 91/93 paired specimens while 90/91 specimens produced negative results in both sample types (Tables 26 and 27). Analyses based on ITD mutations showed positive agreement in 75/77 paired specimens. For TKD mutations, there was positive agreement in 21/22 specimens. Of the TKD mutations, two specimens contained the I836 deletion, which was detected in both sample types. PB and BM concordance (PPA and NPA) was greater than 98% for the point estimate and >95% for the lower bound 95% CI, indicating both sample specimen types can be used for patient diagnosis.

Table 26. Agreement Between PB and BM

	BM	
PB	Positive	Negative
Positive	91	1
Negative	2	90
Total	93	91

Table 27. Agreement Results between PB and BM (PBMAS)

Measure of Agreement	Percent Agreement	95% CI (1)
APA	98.4%	(96.2%, 100.0%)
ANA	98.4%	(96.2%, 100.0%)

(1) The 95% CI was calculated using a non-parametric bootstrapping method.

XII. PANEL MEETING RECOMMENDATION AND FDA'S POST-PANEL ACTION

In accordance with the provisions of section 515(c)(3) of the act as amended by the Safe Medical Devices Act of 1990, this PMA was not referred to the Pathology Panel, an FDA advisory committee, for review and recommendation because the information in the PMA substantially duplicates information previously reviewed by this panel.

XIII. CONCLUSIONS DRAWN FROM PRECLINICAL AND CLINICAL STUDIES

A. Effectiveness Conclusions

The effectiveness of the LeukoStrat® CDx FLT3 Mutation Assay was demonstrated in the bridging study using specimens from the patients screened for the Phase III RATIFY clinical trial (also known as CALGB 10603 or CPKC412A2301) of midostaurin for FLT3-mutated AML and supported the efficacy conclusions with the CTA. The LeukoStrat® CDx FLT3 Mutation Assay supports the accurate detection of newly diagnosed FLT3 mutation-positive AML patient population as enrolled in the A2301 clinical trial. The CTA/CDx concordance for FLT3 status was >97%, with positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) well above the 90% acceptance criteria for positive and negative agreement.

The data from this study support the reasonable assurance of safety and effectiveness of the LeukoStrat® CDx FLT3 Mutation Assay when used in accordance with the indications for use.

B. Safety Conclusions

The LeukoStrat® CDx FLT3 Mutation Assay is not expected to directly cause actual or potential adverse effects, but test results directly impact patient treatment risks. A false negative assay result could cause a patient with FLT3-mutated AML not to experience any potential benefit that might be associated with receiving the RYDAPT® (midostaurin) treatment. However the patient would nevertheless receive intensive chemotherapy as the standard therapy for newly-diagnosed AML. A false positive assay result could cause a patient with FLT3-nonmutated AML who is not expected to benefit from treatment with RYDAPT® (midostaurin) to be treated with this drug. This patient would be subjected to a therapeutic for which no benefit is expected while being exposed to the possible associated risks of receiving the midostaurin (RYDAPT®) treatment.

C. Benefit-Risk Determination

The probable benefits of this device are based on data collected in the clinical Bridging study to the Phase III A2301 clinical trial of midostaurin (RYDAPT®). The assay demonstrated agreement to the A2301 clinical trial assay.

Initial therapy for younger patients with AML has changed little in the past three decades, and generally comprises the '7 + 3' remission induction regimen with cytarabine and daunorubicin, followed by high dose cytarabine for remission consolidation. The 5-year survival rate with this approach is only 30 to 40% in patients under the age of 60 years, and less than 15% in older patients (Stone et al 2005). Further, FLT3-mutated subtypes of AML represent a poor prognosis population of high unmet medical need due to inferior disease-free survival (DFS), inferior overall survival (OS), and higher risk of relapse (with FLT3-ITD specifically) (Kottaridis et al 2001, Whitman et al 2001, Thiede et al 2002). The results of the randomized phase III A2301/RATIFY trial demonstrate the first advance with addition of a targeted agent to traditional chemotherapy in this setting.

Risk/benefit for this FLT3 mutation population is defined on potential clinical impact of false results (false positive and false negative). The supporting clinical validation studies demonstrate PPA (>97%), NPA (>97%) and OPA (>97%) indicating the likelihood of patient misdiagnosis is low. A method comparison of the LeukoStrat® CDx FLT3 Mutation Assay to High throughput Sequencing demonstrating PPA (>92%) and NPA (>96%) also support such a conclusion. Use of the LeukoStrat® CDx FLT3 Mutation Assay shows comparable efficacy results as found in the A2301 clinical trial (HR 0.67, HR 0.77 respectively). Therefore these results support the use of the LeukoStrat® CDx FLT3 Mutation Assay as an aid in selecting patients for FLT3-mutated AML for RYDAPT® (midostaurin) treatment.

RYDAPT® (midostaurin)is a multi-targeted kinase inhibitor, and it has activities against kinases other than FLT3 that are frequently mutated in AML. In addition, in the Phase III study, RYDAPT® (midostaurin) was generally well tolerated. Most of the adverse events (AEs) suspected to be related to study drug, serious adverse events (SAEs), and AEs leading to study discontinuation occurred at similar frequencies in both treatment groups. The only severe or life threatening AEs that occurred more frequently (>5%) in the midostaurin (RYDAPT®) group than in the placebo group were exfoliative dermatitis and device related infections unrelated to the LeukoStrat® CDx FLT3 Mutation Assay.

In conclusion, given the available information above, the data support that for selecting FLT3 mutation positive AML patients using the LeukoStrat® FLT3 Mutation Assay for treatment with midostaurin the probable benefits outweigh the probable risks.

1. Patient Perspectives

   This submission did not include specific information on patient perspectives for this device.

D. Overall Conclusions

The data in this application support the reasonable assurance of safety and effectiveness of this device when used in accordance with the indications for use to detect FLT3 mutations and aid in the assessment of patients for whom midostaurin (RYDAPT®) treatment is being considered. Data from the clinical bridging study to the Phase III A2301 clinical trial of RYDAPT® (midostaurin) demonstrated an overall survival (OS) benefit for midostaurin (RYDAPT®) that appears to be robust and with a clinically-meaningful magnitude for patients with FLT3-mutated AML selected with the LeukoStrat CDx FLT3 Mutation Assay.

XIV. CDRH DECISION

CDRH issued an approval order on April 28, 2017.

The applicant's manufacturing facilities have been inspected and found to be in compliance with the device Quality System (QS) regulation (21 CFR 820).

XV. APPROVAL SPECIFICATIONS

Directions for use: See device labeling.

Hazards to Health from Use of the Device: See device labeling.

Post-approval Requirements and Restrictions: See approval order.

XVI. REFERENCES

None.