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Technical Data Sheet
LeukoStrat® CDx *FLT3* Mutation Assay

For Identification of *FLT3* ITD and TKD Mutations

IVD For *In Vitro* Diagnostic Use

Key to Symbols Used

Symbol	Meaning
IVD	For *In Vitro* Diagnostic Use
REF	Catalog Number
VOL	Reagent Volume
LOT	Lot Number
	Storage Conditions
	Expiration Date
EC REP	Authorized Representative in the European Community
	Manufacturer
	Consult Instructions for Use

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1. Proprietary Name

LeukoStrat® CDx *FLT3* Mutation Assay

2. Intended Use

The LeukoStrat® CDx *FLT3* Mutation Assay is a PCR-based *in vitro* diagnostic test designed to detect internal tandem duplications (ITD) and tyrosine kinase domain (TKD) mutations D835 and I836 in the *FLT3* gene in genomic DNA extracted from mononuclear cells obtained from peripheral blood or bone marrow 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 RYDAPT® (midostaurin) 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.

3. Glossary

3.1. ***FLT3* Mutation Analysis Software (FMA Software)** LeukoStrat® CDx *FLT3* Mutation Assay data analysis software.
3.2. **Internal Tandem Duplication (ITD) Mutation** The duplication and insertion of a portion of the *FLT3* gene that includes the region in and around the juxtamembrane region of the *FLT3* gene.
3.3. **MT Size** In the <u>Summary Results</u> page of the FMA Software, reports the size of the mutant peak in base pair (bp) units.
3.4. **EC** Extraction Control
3.5. **NTC** No Template Control
3.6. **PC** Positive Control
3.7. **Signal Ratio (SR)** Calculated by dividing the mutant peak area by the wild-type peak area.
3.8. **Tyrosine Kinase Domain (TKD) Mutation** Nucleotide change(s) that resulted in changes in codon 835 and/or 836 that are detected by inactivation of the EcoRV restriction digest site within the tyrosine kinase domain of the *FLT*3 gene.

4. Summary and Explanation of the Test

Acute myelogenous leukemia (AML) in general has a poor prognosis. Many studies in AML have shown that the presence of *FLT3* activating mutations portends a poor prognosis.[1,2] The LeukoStrat® CDx *FLT3* Mutation Assay targets regions of the *FLT3* gene to identify internal tandem duplication (ITD) mutations and tyrosine kinase domain (TKD) mutations, such as the D835 and I836 mutations.

The LeukoStrat® CDx *FLT3* Mutation Assay includes reagents, equipment, software and procedures for isolating mononuclear cells and extracting DNA from patient specimens to determine if *FLT3* mutations are present. DNA is amplified via PCR and the amplicons are detected via capillary electrophoresis. The assay measures the ratio of signal from mutation against a background of signal from wild type. A *FLT3* mutation is detected if the mutant:wild-type signal ratio meets or exceeds the cutoff of 0.05.

5. Principles of the Procedure

5.1. **Internal Tandem Duplication (ITD) Mutations of *FLT3***

FLT3 ITD or length mutations 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. ITD mutations result in constitutive autophosphorylation and activation of *FLT3*.

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 this assay, while alleles that contain ITD mutations will produce a product that exceeds 327±1 bp (Figure 1).

5.2. **Tyrosine Kinase Domain (TKD) Mutations of *FLT3***

FLT3 TKD mutations are caused by nucleic acid substitutions and/or deletions that result in a change in the amino acid sequence in this highly-conserved catalytic center. TKD mutations, such as D835 and I836 substitutions and deletions, result in constitutive autophosphorylation and activation of *FLT3*.[2]

Wild-type alleles of the *FLT3* gene include an EcoRV restriction digest site. When a nucleic acid substitution 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 engineered 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 bp or 127±1 bp from the original undigested amplicon product of 145±1 bp or 147±1 bp as measured by this assay (Figure 1).

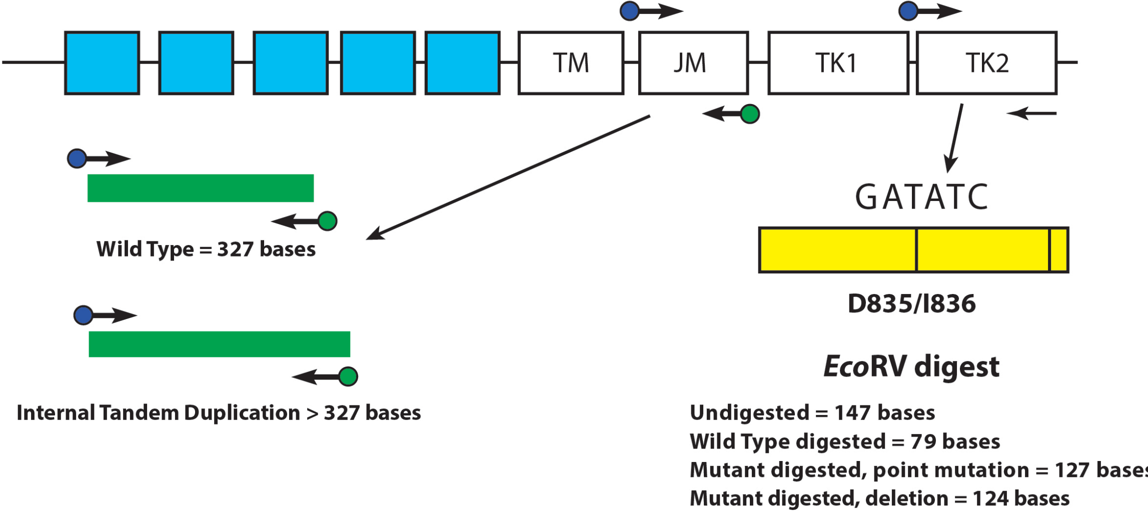


Figure 1. Depicted is a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target the JM region for ITD or the activating loop of the kinase domain for TKD. Colored dots represent fluorophores on labeled primers. The yellow box has vertical black lines that represent the position of the wild type EcoRV restriction digest sites.

6. Reagents and Materials

6.1. The LeukoStrat® CDx *FLT3* Mutation Assay and all reagent vials are usable for one week after opening when stored as described in **Table 1**.

Table 1: Materials Included with LeukoStrat® CDx *FLT3* Mutation Assay, Part Number K4120221

Function	Material Description
MNC Enrichment Materials	**Reagents and materials for separating mononuclear cells from bone marrow or peripheral blood**
DNA Extraction Materials	**Reagents and materials for extracting DNA from mononuclear cells**
PCR Amplification & Capillary Electrophoresis Analysis Materials	***FLT3* ITD Master Mix**
	***FLT3* TKD Master Mix**
	***FLT3* ITD Positive Control**
	***FLT3* TKD Positive Control**
	***FLT3* No Template Control**
	Capillary Electrophoresis Instrument Reagents
Analytical Software	***FLT3* Mutation Assay Analytical Software (FMAS), Version 1.5**

Table 2: Additional Reagents and Materials Required (Not Provided)

Function	Material Description
Cell Counting	**Reagents and materials for use with an automated cell counter.**
DNA Quantification	**Reagents and materials for quantification of DNA.**

Table 3: General Laboratory Materials (Not Provided)

Function	Material Description
Molecular Lab Materials	**General use materials required for performing typical molecular PCR and diagnostic work.**
Cleaning Materials	**General use materials required for cleaning/decontaminating surfaces and equipment in a lab handling DNA.**

7. Instruments/Accessories

NOTE: All equipment should be properly maintained according to the manufacturer's instructions.

- Cell Counter
- DNA Isolation Automator
- Spectrophotometer
- Refrigerator capable of 2 °C to 8 °C storage
- Freezer capable of -15 °C to -30 °C storage
- Dead air box
- Micro pipettes

- Pipette aid
- Repeat pipettes
- Multichannel pipettes, manual and electronic
- Centrifuge capable of 1000 x g with a swing-out rotor and refrigeration
- Centrifuge capable of 1400 x g with a swing-out rotor
- Benchtop mini microcentrifuge
- Benchtop vortex mixer
- Applied Biosystems 3500xL Genetic Analyzer (3500xL), Serial Numbers for US only 21106-231, 23321-020, 23321-030, 25351-081
- With the exception of the genetic analyzers, the above instrumentation are not provided.

7.1. **Software**

- *FLT3* Mutation Analysis Software, Version 1.5
  - URL: fma.invivoscribe.com
  - Operating System: Windows Server2008 R2, IIS
  - Processor: 2 GHz or faster
  - RAM: 2 GB minimum
  - Available Disk Space: 30 GB minimum
  - Web server: IIS that is packaged with Win2008 R2
- EXCEL_0003: *FLT3* Mutation Plate Format Worksheet (Excel workbook)*
- GeneMapper Software
- 3500 Data Collection Software
- Mozilla Firefox Browser, Build 23 or higher

8. Warnings and Precautions

8.1. IVD **This product is for *In Vitro* Diagnostic Use.**

8.2. Caution: Federal law restricts this device to sale by or on the order of a licensed clinical laboratory.

8.3. The assay must be used as a system. Do not substitute other manufacturers' reagents. Do not mix or combine reagents with different lot numbers.

8.4. Materials are stable until the labeled expiration date when stored and handled as directed. Do not use reagents beyond their expiration date.

8.5. Once materials are opened or used for the first time, the date opened is recorded on the material label. The opened expiration date for all materials is one week from the date of opening.

8.6. Laboratory personnel are to wear appropriate personal protective equipment and follow good laboratory practices and universal precautions.

8.7. Due to the analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents or amplification mixtures with samples, controls, or amplified materials. All reagents should be closely monitored for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.

8.8. To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to performing PCR.

8.9. Autoclaving does not eliminate DNA contamination. Work flow in the PCR laboratory should always be in a one way direction between separate work areas; beginning in specimen preparation, then to the

amplification, and finally to detection. Do not bring amplified DNA into the areas designated for specimen preparation.

8.10. All pipettes, pipette tips, and any equipment used in a particular area are to be dedicated to and kept to that area of the laboratory.

8.11. Sterile, disposable plasticware should be used whenever possible to avoid RNase, DNase, or cross-contamination.

8.12. All instruments and equipment must be maintained and calibrated per the manufacturer's recommendations.

9. Specimen Collection and Preparation

9.1. **Precautions**
Biological specimens from humans may contain potentially infectious materials. All specimens should be handled and disposed of in accordance with local standards, regulations, and laws.

9.2. **Interfering Substances**
The following substances are known to interfere with PCR:
9.2.1. Divalent cation chelators
9.2.2. Low retention pipette tips
9.2.3. EDTA (not significant at low concentrations).

9.3. **Specimen Requirements and Handling**
9.3.1. At least 1 mL of peripheral blood and 0.25 mL of bone marrow anti-coagulated with sodium heparin are required for the LeukoStrat® CDx *FLT3* Mutation Assay.
9.3.2. Samples can be stored at 2°C to 8°C for up to 7 days prior to testing.
9.3.3. The LeukoStrat® CDx *FLT3* Mutation Assay has been validated for use with the QIAcube extraction system.
9.3.4. DNA is quantified using a spectrophotometer.
NOTE: If the final quantification value of the extraction control is ≤9.4 ng/µL, the associated DNA samples cannot be tested in the LeukoStrat® CDx *FLT3* Mutation Assay. Reprocess these specimens in order to obtain adequate DNA.
9.3.5. DNA samples may be stored undiluted at -15°C to -30°C for up to one year. Alternatively, DNA samples, undiluted or diluted to 10 ng/µL may be stored at 2°C to 8°C for up to 7 days.
NOTE: Undiluted DNA can be exposed to up to 5 freeze/thaw cycles.
9.3.6. DNA samples ≥10.5 ng/µL must be diluted to 10 ng/µL in AE buffer using non-binding surface tubes.
9.3.7. Amplification, detection, and capillary electrophoresis are conducted according to the instructions described in the LeukoStrat® CDx *FLT3* Mutation Assay IFU (Document #: 280374).

10. Retesting

10.1.1. Up to 4 retests for a single control/sample when sample is invalid were conducted in the studies described below. It is recommended that a new sample is obtained if retesting exceeds this.

11. Interpretation of Results

11.1. 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 signal ratio is the peak

area of the mutant signal, if present, divided by the peak area of the wild type signal, if present. The mutant:wild-type signal ratio is displayed to two decimal places.

11.2. To note, ITD mutations may carry multiple mutations; the peak areas of the mutations are summed to calculate the total mutant signal. Furthermore, a sample may contain no wild type-signal (pure mutant). In this case the mutant:wild-type signal ratio is reported by the *FLT3* Mutation Analysis Software as **100.00**; it is not intended to convey a ratio value.

11.3. If the mutant:wild-type signal ratio for either ITD or TKD in a valid sample result is at or above the cut-off of 0.05, the result will be interpreted and reported as **Positive** (mutation detected).

11.4. If the mutant:wild-type signal ratios for both ITD and TKD in a valid sample result are below the cut-off of 0.05, the result will be interpreted and reported as **Negative** (mutation signal below clinical cut-off).

11.5. The mutation status of a sample is defined by the rules included in **Table 4**.

Table 4. Determining Sample 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

11.6. If Fail Details are provided in the *FLT3* Mutation Analysis Software report, repeat the run or retest samples according to instructions in the Retesting section.

12. Limitations of Procedure

12.1. Test only the indicated specimen types, as the LeukoStrat® CDx *FLT3* Mutation Assay has been validated for use only with peripheral blood and bone marrow aspirate. Reliable results are dependent on appropriate storage and processing of the specimens; therefore, follow the procedures in this Package Insert.

12.2. The LeukoStrat® CDx *FLT3* Mutation Assay has been validated using only the included QIAamp DSP DNA Blood Mini Kit to extract genomic DNA.

12.3. The LeukoStrat® CDx *FLT3* Mutation Assay will detect ITD mutations sized 3 bp to 323 bp; however, the assay is only validated to detect mutations sized 30 bp to 279 bp.
- 12.3.1. ITD insertions sized between 3 bp and 30 bp will be reported as ITD mutations.
- 12.3.2. ITD insertions sized between 279 bp and 323 bp will be reported as ITD mutations.
- 12.3.3. ITD insertions sized greater than 323 bp, will not be reported as insertions.

12.4. This assay may not detect *FLT3* mutations that present below the sensitivity level of the assay.
- 12.4.1. For ITD insertions sized 30 bp to 126 bp, inclusive, an allelic ratio of 0.08 will yield a positive LeukoStrat® CDx *FLT3* Mutation Assay result.
- 12.4.2. For ITD insertions sized 129 bp to 279 bp, inclusive, an allelic ratio of 1 will yield a positive LeukoStrat® CDx *FLT3* Mutation Assay result.
- 12.4.3. For TKD mutations that destroy the EcoRV site, an allelic ratio of 0.18 will yield a positive LeukoStrat® CDx *FLT3* Mutation Assay result.

12.5. The results of the assay should always be interpreted in the context of clinical data and other tests performed for the patients.

12.6. Detection of a mutation is dependent on the number of mutant sequence copies present in the specimen and may be affected by specimen integrity, amount of DNA isolated, and the presence of interfering substances. PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA and other agents.

12.7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the LeukoStrat® CDxLeukoStrat® *FLT3* Mutation Assay.

12.8. The LeukoStrat® CDxLeukoStrat® *FLT3* Mutation Assay is a qualitative test. The test is not for quantitative measurements of ITD or TKD mutations.

12.9. The allelic ratio of a specimen cannot be calculated, measured, or determined using this assay.

13. Non-Clinical Performance Evaluation

13.1. **<u>All Evaluable Set:</u>**

13.1.1. 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 which 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 5. Agreement with and without the invalid results is shown in Table 6.

Table 5. 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 6. 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%)

***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.

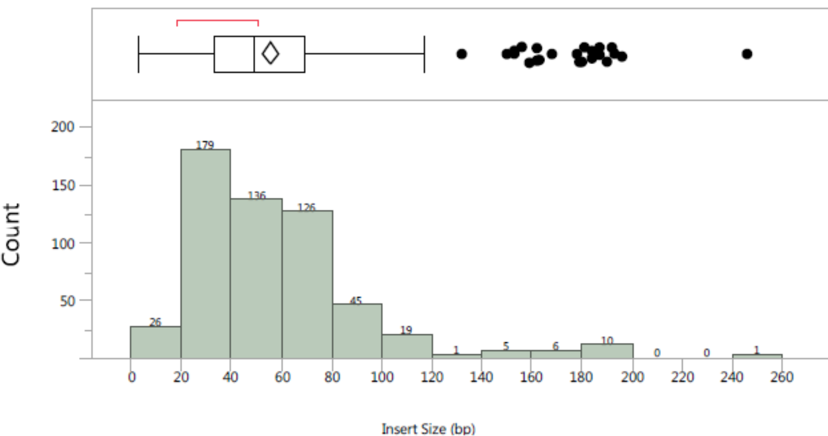


Figure 2: Distribution of ITD Insert Sizes by CDx (381 Positive Samples, N Insert Sizes = 554, Mean=55.6).

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 7). Without the CDx invalids, the PPA, NPA and OPA are at or above 88%.

Table 7. Agreement on ITD between CDx and 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 Table 8.

Table 8. Agreement on TKD between CDx and 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 were analyzed separately for peripheral blood and bone marrow and demonstrated to be comparable .

13.2. **Analytical Sensitivity – Limit of Blank (LoB)**

13.2.1. When samples containing wild type DNA only (i.e. a mutant blank) were tested in the LeukoStrat® CDx *FLT3* Mutation Assay, the SR was 0.00 in the ITD assay and 0.00 to 0.01 in the TKD assay. This limit of blank is well below the clinical cutoff SR of 0.05.

13.3. **Analytical Sensitivity**

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 9. Additional cell lines 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. 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 (Table 9). 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 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).

Table 9. 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
TKD CS1	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)	-
TKD CS2	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)	-
ITD CS1	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)	-
ITD CS2	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)	-
ITD CS3	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	-

Sample ID	Mutation	Level	TSR	SR Mean	AR of Blend	Valid N	N (%) SR > LoB	N (%) SR > 0.05	*Classification
		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
TrueNegTKD	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.

13.3.1. The LeukoStrat® CDx *FLT3* Mutation Assay is capable of detecting the following mutant/wild-type allelic ratios of the following mutation types:

13.3.1.1. For ITD insertions sized at 24 bp, an allelic ratio of 0.107 was detected above the LoB SR in more than 95% of samples. The SR %CV for these samples was 7.1%.

13.3.1.2. For ITD insertions sized at 66 bp, an allelic ratio of 0.189 was detected above the LoB SR in more than 95% of samples. The SR %CV for these samples was 7.1%.

13.3.1.3. For ITD insertions sized at 279 bp, an allelic ratio of 0.539 was detected above the LoB SR in more than 95% of samples. The SR %CV for these samples was 25.6%.

13.3.1.4. For D835 TKD mutations that destroy the EcoRV site, an allelic ratio of 0.089 was detected above the LoB SR in more than 95% of samples. The SR %CV for these samples was 4.5%.

13.3.1.5. For I836 TKD mutations that destroy the EcoRV site, an allelic ratio of 0.144 was detected above the LoB SR in more than 95% of samples. The SR %CV for these samples was 5.7%.

13.3.1.6. Conversion of AR values to % Mutant is shown in Table below

Table 10. 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

13.4. **Precision**

13.4.1. The precision of the LeukoStrat® CDx *FLT3* Mutation Assay was determined by three operators independently testing 10 replicates each of ITD mutation samples with inserts ranging in size from 30 bp to 126 bp and TKD mutation samples. The 10 replicates were tested in batches of two 5 separate times.

13.4.2. For the ITD mutation samples, the SR %CV ranges for the 3 operators were 7.4% to 15.0%, 3.7% to 13.0%, and 4.2% to 8.8%.

13.4.3. For the TKD mutation samples, the SR %CV ranges for the 3 operators were 6.3% to 11.2%, 5.8% to 9.3%, and 5.5% to 8.3%.

13.5. **Operator-to-Operator Reproducibility (cell-lines)**

13.5.1. Samples consisted of ITD cell lines 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. Three operators using one reagent lot and one instrument over 15 runs tested 10 replicates each the SR %CV ranged from 6.6% to 13.3%.

13.5.2. For TKD mutation samples, the SR %CV ranged from 7.9% to 9.3%.

13.5.3. For ITD clinical specimens up to 90 bp inserts, the SR %CV ranged from 4.4% to 16.1%.

13.5.4. For the ITD clinical specimens sized at 217 bp, the SR %CV ranged from 26.9% to 27.2%. Thus, ITD mutations at 217 bp may exhibit higher SR variability.

13.6. **Operator-to-Operator Reproducibility (clinical samples)**

13.6.1. In a second study, precision 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.

13.6.2. The total %CV of all mutation types and levels are shown in table below 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% (Table 11). 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 11. 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
S1	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
		MP	0.132	0.002 (6.67%)	0.003 (17.43%)	0.005 (75.90%)	0.006	4.6
S2	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
S3	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 (14.46%)	0.000 (0.00%)	0.005 (85.54%)	0.006	4.4
S4	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
S5	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
S6	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
S7	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

13.7. **Lot-to-Lot and Instrument-to-Instrument Reproducibility**

13.7.1. The lot-to-lot and instrument-to-instrument reproducibility was determined by a single operator testing the same set of samples using 3 lots of reagents on 3 sets of instruments. Cell line samples consisted of ITD samples containing inserts ranging in size from 30 bp to 126 bp and TKD mutation samples.

13.7.2. For the ITD mutation samples, the SR %CV ranged from 3.0% to 8.4%.

13.7.3. For the TKD mutation samples, the SR %CV ranged from 5.4% to 10.6%.

13.8. **Interfering Substances – Exogenous**

13.8.1. The LeukoStrat® CDx *FLT3* Mutation Assay is capable of detecting ITD mutations sized 18 bp to 114 bp and TKD mutations in the presence of Na-Heparin and the wash buffer used during the DNA isolation process.

13.9. **Interfering Substances – Endogenous**

13.9.1. The LeukoStrat® CDx *FLT3* Mutation Assay is capable of detecting ITD mutations sized 18 bp to 114 bp and TKD mutations in the presence of lipids/triglycerides, hemoglobin, protein, and bilirubin.

13.10. **Interfering Substances – Treatment Drugs**

13.10.1. The LeukoStrat® CDx *FLT3* Mutation Assay is capable of detecting ITD mutations sized 18 bp to 114 bp and TKD mutations in the presence of cytarabine and daunorubicin.

13.11. **Carryover and Cross Contamination**

13.11.1. When challenged via the typical checkerboard plate map set ups it was shown that carryover and cross contamination were not problematic for the LeukoStrat® CDx *FLT3* Mutation Assay:

13.11.1.1. Carryover / Cross Contamination detected was 0%.

13.11.1.2. ITD and TKD No Template Control failure rate was 0%.

13.11.1.2.1. **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 long ITD cell line. 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.

14. Clinical Performance Evaluation

14.1. **Pivotal Bridging Study Overview**

14.1.1. 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 A2301 Clinical Trial Assay (CTA) and the LeukoStrat® CDx *FLT3* Mutation Assay in the intent-to-test population. This pivotal Bridging Study for the LeukoStrat® CDx *FLT3* Mutation Assay, corresponds to the Phase III CPKC412A2301 (A2301, CALBG 10603, RATIFY) clinical study of midostaurin in newly diagnosed AML patients with

FLT3 mutations. The LeukoStrat® CDx *FLT3* Mutation Assay is intended to assist physicians in making treatment decisions for their AML patients with *FLT3* Mutations.

14.1.2. The LeukoStrat® CDx *FLT3* Mutation Assay has been developed by Invivoscribe as a companion diagnostic to be used as an aid in the assessment of AML patients for whom midostaurin treatment is being considered. Agreement to the CTA and drug efficacy when stratified by the LeukoStrat® CDx *FLT3* Mutation Assay was evaluated in this Bridging Study. Additional assessment of bone marrow and peripheral blood agreement and CTA/CDx comparison to an independent test method was completed.

14.2. **Study Objectives**

14.2.1. Primary objectives of the study were to 1) establish agreement with respect to selection of *FLT3* mutant patients between the A2301 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).

14.2.2. Secondary objectives of the study were to 1) identify potential demographic and disease state covariates affecting the relationship between diagnostics and efficacy and 2) to present objective evidence that gDNA isolated from mononuclear cells (MNCs) isolated from either bone marrow (BM) or peripheral blood (PB) provide concordant results from both specimen types for the LeukoStrat® CDx *FLT3* Mutation Assay through comparison of paired samples.

14.2.3. Other testing and analyses included the assessment of the presence or absence of *FLT3* mutation by next generation DNA sequencing using the high throughput DNA sequencing technology as an independent source of sequence information.

14.3. **Patient Population**

14.3.1. Over 3000 patients were screened with the clinical trial assay in order to enroll 717 patients into the A2301 trial. Testing was performed using a common testing protocol at 9 designated testing laboratory sites. Patients were enrolled in the A2301 trial based on identification of *FLT3* mutations in a BM or PB sample. The clinical cut-off of the test for the trial was set at 0.05 (mutant:WT signal ratio).

14.3.2. The Concordance Analysis Set (CAS) (N = 1100) included a subset of patients who provided informed consent and were tested with the CDx test. The agreement analysis between the CTA test and the CDx test used the CAS population. For patients with both BM and PB samples available, the CDx results from the bone marrow sample were used in the statistical analysis, as defined in the Bridging Study protocol.

14.4. **Selection of Patients and Aliquots for *FLT3* CDx Testing**

14.4.1. The patient set for the Bridging study was selected from the available banked samples and with the informed consent information available at the time. Samples from 618 enrolled patients (CTA+) were available. An equal number of unenrolled patients (presumed CTA-) were also selected, with an additional 15% overage to allow for the potential of positive *FLT3* mutation results among the unenrolled patients.

The selected Bridging Study was comprised of 503 CTA positive enrolled patient specimens and 555 CTA negative specimens.

Once patients were identified, one sample aliquot was selected per patient. Among patients having bone marrow and peripheral blood samples (110 enrolled patients and 123 unenrolled patients), one aliquot from each sample type was selected to support the comparison of sample types. For patients having both sample types, the bone marrow assay result was used for the CTA/CDx concordance and clinical efficacy analyses.

14.5. **Safety Analysis**

14.5.1. The LeukoStrat® CDx *FLT3* Mutation Assay is not expected to directly cause actual or potential adverse effects, but test results may directly impact patient treatment risks.

14.6. **Effectiveness**

14.6.1. Primary CDx Clinical Validation analyses were performed using samples from 1058 AML patients from the A2301 study population. The LeukoStrat® *FLT3* Mutation CDx Assay demonstrated agreement to the CTA, similar efficacy to A2301 and accuracy compared to high throughput sequencing (Table 12).

14.6.1.1. The primary analysis demonstrated:

The CTA/CDx agreement for *FLT3* status was high (>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.

Table 12. Overall Agreement Table between CDx and CTA

FLT3 CDx	**FLT3 CTA**	
	+	-
+	489	8
-	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.

Agreement (95% CI) are:

- PPA 97.2% (95.4%, 98.5%)
- NPA 97.3% (95.6%, 98.5%)
- OPA 97.3% (96.1%, 98.2%)

The CTA/CDx agreement for the individual ITD and TKD tests (PPA, NPA, OPA) was above 90% (Table 13 and Table 14).

Table 13. Agreement Table between ITD CDx and ITD CTA

	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.

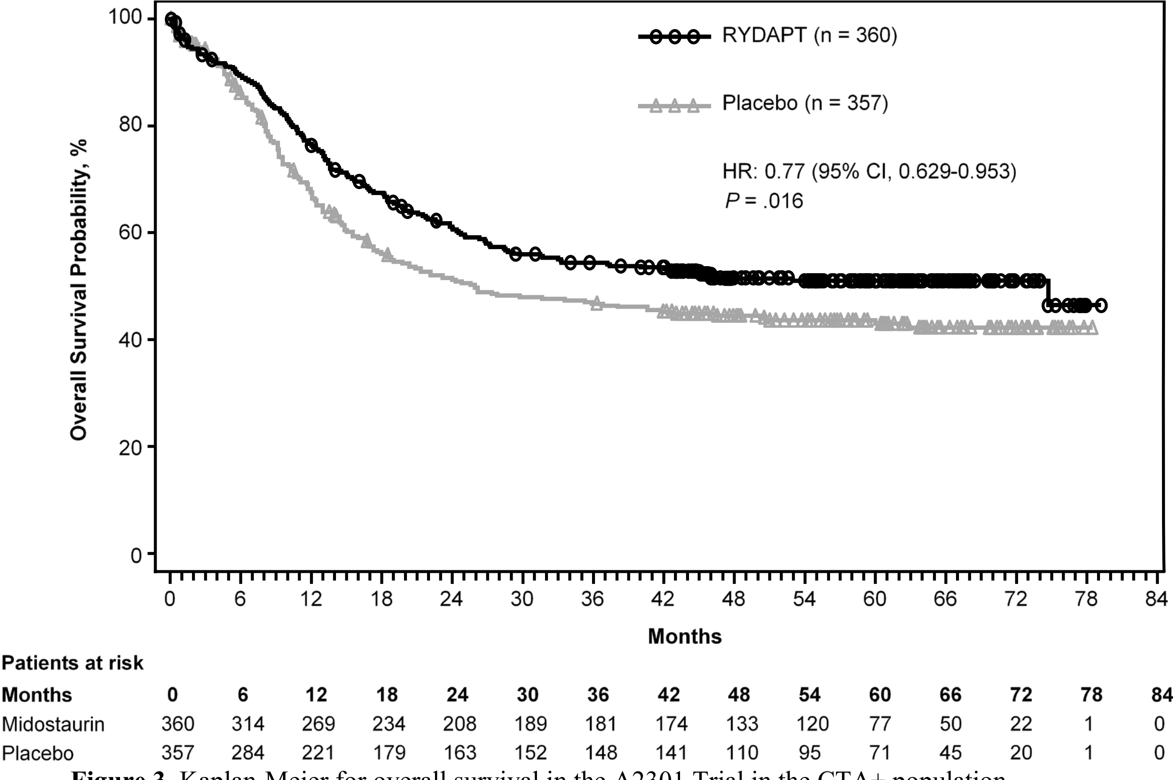
Table 14. Agreement Table between TKD CDx and TKD CTA

	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.

In the clinical trial, efficacy was established on the basis of overall survival (OS) using the CTA, 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 3). Because survival curves plateaued before reaching the median, median survival could not be reliably estimated.



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

Figure 3. Kaplan-Meier for overall survival in the A2301 Trial in the CTA+ population.

14.6.1.2. 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 overall A2301 clinical trial results and the CDx tested subset and for both overall survival with non-censoring at stem cell transplant (See Figure 3 and Figure 4, respectively). Hazard ratio (95% CI) outcomes for OS were 0.67 (0.52, 0.87) vs A2301 OS 0.77 (0.63, 0.95).

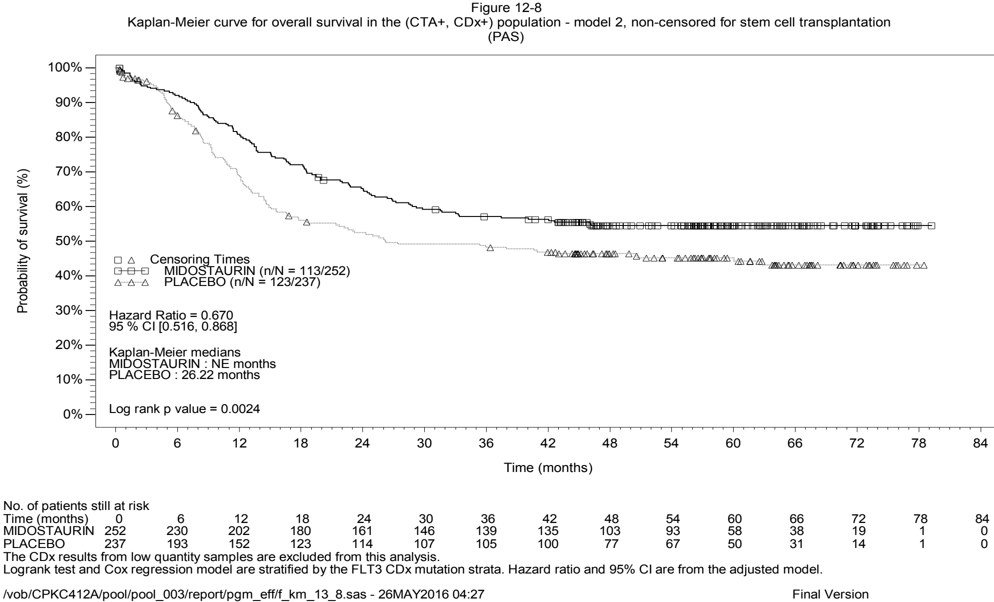


Figure 4 Kaplan-Meier for overall survival in the A2301 Trial in the CTA+, CDx+ Population.

14.6.1.3. The secondary and other analyses demonstrated:

Clinically important demographic and prognostic features such as leukemia cytogenetics were well balanced between the CDx-evaluable and the CDx-unevaluable populations as well as the treatment and placebo arms.

Peripheral blood and bone marrow concordance (PPA and NPA) was greater than 95%, indicating both sample specimen types can be used for patient diagnosis (Table 15 and Table 16).

Table 15. Agreement Table Between Peripheral Blood and Bone Marrow

	Bone marrow	
Peripheral blood	+	-
+	91	1
-	2	90
Total	93	91

Table 16. Agreement Between Peripheral Blood and Bone Marrow

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		

14.7. **Conclusions**

14.7.1. Overall these results support that the LeukoStrat® CDx *FLT3* Mutation Assay identifies the same AML patient population as enrolled in the A2301 clinical trial with respect to *FLT3* ITD and TKD gene mutations.

14.7.2. 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.

15. References

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16. Technical and Customer Service

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