LeukoStrat®

Instructions for Use

LeukoStrat® *FLT3* Mutation Assay 2.0

CE UKCA IVD

For identification of fms related tyrosine kinase 3 (*FLT3*) internal tandem duplication (ITD) mutations and tyrosine kinase domain (TKD) mutations.

IVD For *in vitro* diagnostic use.

invivoscribe®

Storage Conditions: **-85ºC to -65ºC**
(DNA controls may be separated from assay kits and stored at 2°C to 8°C)

Catalog #	Products	Quantity
REF 94120091	LeukoStrat *FLT3* Mutation Assay 2.0 – ABI Fluorescence Detection	33 Reactions

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1. Intended Use

The LeukoStrat *FLT3* Mutation Assay 2.0 is an *in vitro* diagnostic product intended for PCR-based detection of *FLT3* activating mutations in patients with acute myelogenous leukemia (AML).

Specifically, the *FLT3* Mutation Assay 2.0 can be used to:

- Identify internal tandem duplications (ITD) in the *FLT3* gene.
- Identify tyrosine kinase domain (TKD) mutations in the *FLT3* gene.

2. Summary and Explanation of the Test

2.1. Background

Acute myeloid leukemia (AML) in general has a poor prognosis. Assessment of the mutation status of the *FLT3* (fms related tyrosine kinase 3) receptor gene in karyotype normal AML is the most important prognostic indicator of disease outcome, which is often substantial, as many studies in AML have shown that the presence of *FLT3* activating mutations portends a poor prognosis.[1, 2] For this reason *FLT3* activation mutation testing is required to stratify disease and determine appropriate treatment options.

2.2. Summary

This LeukoStrat 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. Using this assay, DNA is amplified via PCR with fluorophore labeled primers, TKD amplicon is enzymatically digested, and the amplicons are detected via capillary electrophoresis. This test kit includes two (2) PCR master mixes, along with positive and negative controls for mutant detection. Each master mix (*FLT3* ITD Master Mix and *FLT3* D835 Master Mix) contains a fluorophore-labeled PCR primer set for the respective detection of internal tandem duplication mutations or TKD region mutations.

This assay reliably detects *FLT3* mutations comprising greater than or equal to 5% of the total cell population. Always interpret the results of molecular mutation tests in the context of clinical, histological and immunophenotypic data.

3. Principles of the Procedure

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

FLT3 internal tandem duplication 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*.[1] When interrogating human gDNA template on an ABI 3500xL instrument platform, wild-type *FLT3* alleles will amplify and produce a 327 base pairs (bp) product using this assay, while alleles that contain ITD mutations will generate a product that exceeds ≥327 bp (Figure 1).

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

FLT3 tyrosine kinase domain (TKD) mutations are caused by nucleic acid substitutions that result in a change in the amino acid sequence in this highly conserved catalytic center. TKD mutations, such as D835 and I836, 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 *FLT3* target region is amplified using PCR and then an EcoRV restriction digest is performed. One of the PCR primers 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. When interrogating human gDNA template on an ABI 3500xL instrument platform, wild-type alleles of the *FLT3* gene yield digestion products of 79±1 bp whereas mutant alleles yield products of 124+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). Product lengths correspond to results obtained through the use of GeneScan™ - 600™ LIZ Size Standard v2.0 and the ABI3500xL instrument. The use of different size standards and instruments may yield different product sizes.

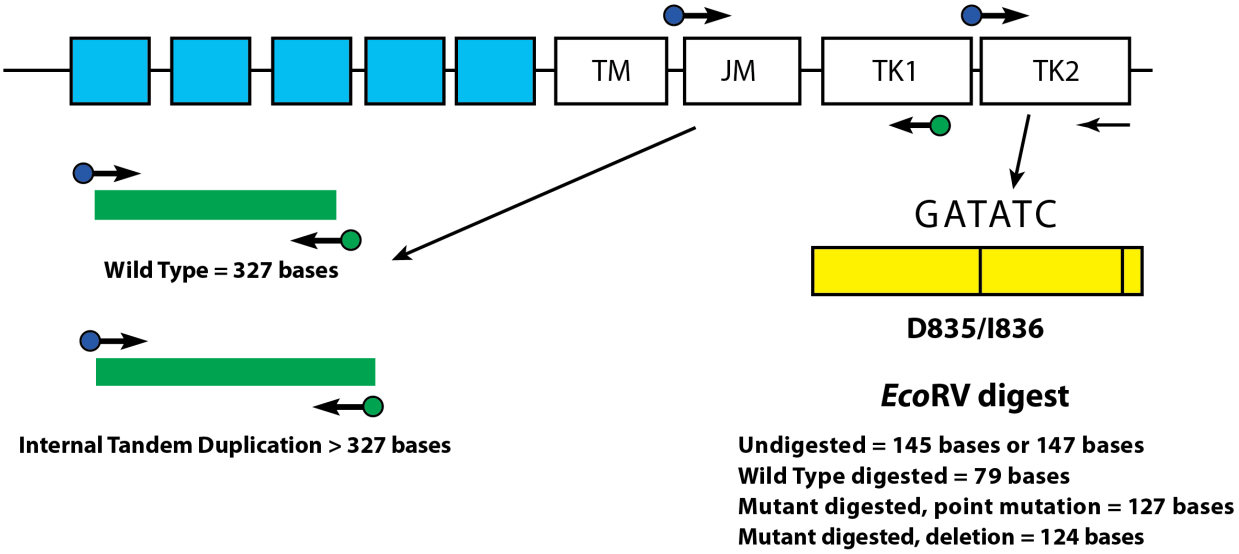

Figure 1. Depicted is a representation of the *FLT3* JM region and the activating loop of the kinase domain. Green and blue dots with black arrows represent the relative positions of primers that target the JM region for ITD and the remaining blue dot and black arrows represent the relative positions of the primers that target TKD mutations in the activating loop of the kinase domain. The yellow box has vertical black lines that represent the position of the wild-type EcoRV restriction digest sites. **Product sizes reflect human gDNA templates measured on an ABI 3500xL Genetic Analyzer.**

3.3. Differential Fluorescence Detection

Differential fluorescence detection is commonly used to resolve the different-sized amplicon products using a capillary electrophoresis instrument. Primers can be conjugated with several different fluorescent dyes (fluorophores) so that they can produce different emission spectra upon excitation by a laser in the capillary electrophoresis instrument. In this manner, different fluorescent dyes can correspond to different targeted regions. This detection system results in high sensitivity, single nucleotide resolution, differential product detection, and relative quantification. In addition, the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide, can virtually be eliminated. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. Inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately 1 to 2 nucleotides. This reproducibility and sensitivity coupled with the automatic archiving of specimen data allows for the monitoring, tracking, and comparison of data from individual patients over time.

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog #	Description	Quantity
REF 94120091	LeukoStrat *FLT3* Mutation Assay 2.0 – ABI Fluorescence Detection	33 Reactions

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	# of Units	Storage Temperature
Master Mix	REF 24120011CE	***FLT3* ITD Master Mix – 6FAM & HEX** Fluorophore-labeled oligonucleotides targeting the *FLT3* gene in a buffered salt solution.	1500 µL	1	-85°C to -65°C
	REF 24120031CE	***FLT3* D835 Master Mix – 6FAM** Fluorophore-labeled oligonucleotides targeting the *FLT3* TKD region in a buffered salt solution.	1500 µL	1	
Positive Control DNAs	REF 40883390	***FLT3* ITD Positive Control** 50 µg/mL of DNA in 1/10th TE solution	100 µL	1	2°C to 8°C or -85°C to -65°C
	REF 40883400	***FLT3* D835 Positive Control** 50 µg/mL of plasmid DNA in 1/10th TE solution	100 µL	1	
Negative (Normal) Control DNA	REF 40920030	***FLT3* Negative Control** 50 µg/mL of DNA in 1/10th TE solution	100 µL	1	

4.2. Warnings and Precautions

IMPORTANT! Please read the Instructions for Use carefully prior to starting the assay procedure and follow each step closely.

- IVD This product is for *in vitro* diagnostic use.
- Use this assay kit as a system. Do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes, or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Close adherence to the protocol will assure optimal performance and reproducibility. Ensure use of correct thermal cycler program, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
- Use only EcoRV for the restriction enzyme digest, use of the incorrect restriction enzyme can yield false positive or negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Perform laboratory procedures with standard personal protective equipment (gloves, laboratory coats and protective eye wear). Follow good laboratory practices and universal precautions when working with specimens. Handle specimens in approved biological safety containment facilities and opened only in certified biological safety cabinets.
- Use molecular biology grade water with the preparation of specimen DNA.
- Due to the analytical sensitivity of this test, take extreme care to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. Closely monitor reagents for signs of contamination (*e.g.,* negative controls giving positive signals). Discard reagents suspected of contamination.
- To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to doing PCR.
- Follow uni-directional work flow in the PCR laboratory: begin with master mix preparation, move to specimen preparation, then to amplification, and finally to detection.
- Autoclaving does not eliminate DNA contamination.
- Do not bring amplified DNA into the areas designated for master mixes or specimen preparation.
- Dedicate all pipettes, pipette tips, and any equipment used in a particular area to that area of the laboratory
- Non-disposable items must be decontaminated in 10% bleach and rinsed with distilled water two separate times before returning them to the starting areas. Use sterile, disposable plasticware whenever possible to avoid contamination.

4.3. Storage and Handling

- For any duration other than immediate use, store assay kits at **-85°C to -65°C**.
- The optimum storage temperature for DNA controls is 2°C to 8°C, but DNA controls can be stored at -85°C to -65°C long term.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are resuspended completely. Excessive vortexing may shear DNA and cause labeled primers to lose their fluorophores.
- Store PCR master mixes in the dark to protect fluorophore labeled primers.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Due to high salt concentrations, PCR master mixes are sensitive to freeze/thaw cycles. Aliquot master mixes into sterile O-ring screw-cap tubes if necessary.

5. Instruments

5.1. Thermal cycler

- Use or function: Amplification of DNA samples
- Suggested instrument: Veriti™ Dx thermal cycler or equivalent
- Performance characteristics and Specification:
  - Minimum thermal range: 15°C to 96°C
  - Minimum ramping speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.
- See section 7.4. *Amplification* for thermal cycler program.

5.2. ABI 3130/3130xl or 3500/3500xL

- Use or Function: Fragment detection and analysis
- The following capillary electrophoresis instruments will meet the performance needs for this assay:
  - ABI 3130 Genetic Analyzer* (4-capillaries)
  - ABI 3130xl Genetic Analyzer* (16-capillaries)
  - ABI 3500 Genetic Analyzer*(8-capillaries)
  - ABI 3500xL Genetic Analyzer* (24-capillaries)
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.
- Calibrate the Genetic Analyzers with the DS-33 Matrix Standard for Dye Set G5 (recommended). DS-30 Matrix Standard for Dye Set D can be used with the 3130 series (alternate option).
- Use the default settings for your polymer and capillary type.
- See section 7.6. *ABI Fluorescence Detection*.

***Warning:** These are not CE-marked products.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle specimens in accordance with your institute's Bloodborne Pathogen program and/or Biosafety Level 2.

6.2. Interfering Substances

The following substances are known to interfere with PCR:

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)
- Heparin
- Cells suspended in a fixative, such as acetic acid, B5, etc.

6.3. Specimen Requirements and Handling

This assay tests **genomic DNA** (gDNA) from the following sources:

- Peripheral blood or bone marrow aspirate anti-coagulated with heparin, EDTA, ACD or previously isolated mononuclear cells that are fresh in an appropriate media (RPMI or similar) or frozen in an appropriate cryopreservation media.
- Peripheral blood and bone marrow aspirates may be stored at 2°C to 8°C for 7 days and still give valid results. Isolated mononuclear cells may be stored fresh for up to 7 days or indefinitely if properly cryopreserved.
- 500 ng of gDNA (stored at 2°C to 8°C or below -15°C and shipped at ambient temperature, cool conditions, or on dry ice).

6.4. Sample Preparation

Extract the gDNA from patient specimens as soon as possible. DNA samples are standardized to a final concentration of 50 µg/mL.

6.5. Sample Storage

Store gDNA at 2°C to 8°C or below -15°C.

7. Assay Procedure

7.1. Materials Provided

See Table 3 for materials provided in each kit.

Table 2: Kit components

Catalog #	Description
REF 24120011CE	*FLT3* ITD Master Mix – 6FAM & HEX
REF 24120031CE	*FLT3* D835 Master Mix – 6FAM
REF 40883390	*FLT3* ITD Positive Control
REF 40883400	*FLT3* D835 Positive Control
REF 40920030	*FLT3* Negative Control

7.2. Materials Required (not provided)

Table 3: Materials required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog #	Notes
DNA Polymerase	Roche®: • EagleTaq™ DNA Polymerase Invivoscribe, Inc. • FalconTaq DNA Polymerase or equivalent	05206944190 60970130	N/A
Glass Distilled De-ionized Molecular Biology Grade or USP Water	N/A	N/A	Sterile and free of DNase and RNase.
Calibrated Pipettes	N/A	N/A	Must be able to accurately measure volumes between 1 µL and 1000 µL.
Thermal cycler	ThermoFisher Scientific: • Veriti™ Dx Thermal Cycler or equivalent	4452300	N/A
EcoRV endonuclease	New England Biolabs: • EcoRV 20,000 units/mL	R0195S or R0195L	N/A
NEBuffer	New England Biolabs: • NEBuffer 3.1	B7203S	N/A
Vortex Mixer	N/A	N/A	N/A
PCR plates or tubes	N/A	N/A	Sterile
Filter barrier pipette tips	N/A	N/A	Sterile, RNase/DNase/Pyrogen-free
Microcentrifuge tubes	N/A	N/A	Sterile
ABI Capillary Electrophoresis Instrument	ThermoFisher Scientific: • ABI 3130 Genetic Analyzer series • ABI 3500 Genetic Analyzer series	313001R or 3130XLR 4406017 or 4406016	N/A
Hi-Di Formamide	ThermoFisher Scientific: • Hi-Di™ Formamide	4440753	N/A
Size Standards	ThermoFisher Scientific: • Recommend for ABI 3130 and ABI 3500 series: ○ GeneScan 600™ LIZ® dye Size Standard v2.0	4408399	N/A

Table 3: Materials required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog #	Notes
Size Standards	ThermoFisher Scientific: • Alternate for ABI 3130 series: ○ GeneScan - 400HD ROX™ dye Size Standard	402985 or 4310366	N/A
Spectral Calibration Dye Set D or G5	ThermoFisher Scientific: • Recommend for ABI 3130 and ABI 3500 series: ○ DS-33 Matrix Standard Kit (Dye Set G5) • Alternate option for ABI 3130 series: ○ DS-30 Matrix Standard Kit (Dye Set D)	4345833 4345827	N/A
Polymer	ThermoFisher Scientific: • POP-7™ Polymer: ○ POP-7 for 3130 series ○ POP-7 for 3500 series	4352759 or 4363785 4393708 or 4393714 or A26073	N/A
Buffer	ThermoFisher Scientific: • For ABI 3130 series: ○ 310 and 31xx Running Buffer, 10x • For ABI 3500 series: ○ Anode Buffer Container 3500 Series ○ Cathode Buffer Container 3500 Series	402824 4393927 4408256	N/A
96-well aluminum foil sheet	N/A	N/A	N/A
96-well 8-cap strips	N/A	N/A	N/A

7.3. Reagent Preparation

Optional: Test all unknown samples with the Specimen Control Size Ladder master mix to ensure that no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result. The Specimen Control Size Ladder Master Mix can be purchased separately from Invivoscribe (REF: 20960021 for ABI detection).

- **Test positive**, **negative** and **no template** controls with appropriate master mix(es).

Amplification Mixes

7.3.1. Clean a dead air box with 10% bleach followed by distilled water, repeat with 70% ethanol.

7.3.2. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely (avoid direct exposure to light); then gently vortex or invert tube 5-10 times to mix.

7.3.3. In containment hood or dead air box, remove an appropriate aliquot from each master mix to individual clean, sterile microcentrifuge tubes.

- Aliquot volumes are 45 µL for each reaction.
- Include an additional reaction volume for every 15 reactions to allow for pipetting error.
- Thus, for each master mix, the number of reactions (n) should be:

n =	**1 × # of samples**	
	+ 1	positive control DNA (***FLT3* Positive Control**)
	+ 1	negative control DNA (***FLT3* Negative Control**)
	+ 1	no template control (water)
	+ 1	to allow for pipetting error
n = # of samples + 4 Total		

- Therefore, the total aliquot volume for each master mix is **n × 45 µL**.

7.3.4. Add 1.25 units (or 0.25 µL at 5 U/µL) of Taq DNA polymerase per reaction to each master mix.

- The total Taq DNA polymerase added to each master mix is **n × 0.25 µL**.
- Invert tube several times to adequately mix.

7.3.5. For each reaction, aliquot 45 µL of the appropriate master mix + DNA polymerase solution into individual wells in a PCR plate or tube.

7.3.6. Add 5 µL of appropriate template (sample DNA at a concentration of 50 µg/mL, positive control DNA, negative control DNA, or water) to the individual wells containing the respective master mix solutions.

- Pipette up and down 5-10 times to mix.

7.3.7. Seal the PCR plate.

- Samples are now ready for PCR amplification.

Table 4: Reaction set-up

Reagent	Volume
Master Mix + EagleTaq DNA Polymerase	45 µL
Sample or Control DNA	5 µL
Total Volume	**50 µL**

7.4. Amplification

7.4.1. Amplify the samples using the following PCR program:

Table 5: PCR program. Apply a 75% ramp rate when using the Veriti thermal cycler.

Step	Temperature	Time	Cycles
1	95°C	5 minutes	1
2	94°C	30 seconds	35x
3	55°C	30 seconds	
4	72°C	60 seconds	
5	72°C	60 minutes	1
6	4°C	∝	1

7.4.2. Remove the amplification plate or tubes from the thermal cycler.

7.4.3. Store amplicons at 2°C to 8°C until ready for analysis on the ABI 3130/3130xL or ABI 3500/3500xL.

- Detection must be within 30 days of amplification.

7.5. Restriction Digest for *FLT3* D835 Master Mix Only

7.5.1. Using gloved hands, remove the 10X NEBuffer 3.1 from the freezer. Allow it to thaw completely; then gently vortex to mix.

7.5.2. In containment hood or dead air box, add the following to an individual clean, sterile microcentrifuge tube.

- To allow for pipetting error, include an additional reaction volume for every 15 reactions.
- Thus, the number of reactions (**n**) is:

n = **1 × # of samples**
+ 1 to allow for pipetting error
n = # of samples + 1 Total

7.5.3. Add 15.7 µL of molecular grade water per reaction to the tube.

- The total volume of water added is **n × 15.7 µL**.

7.5.4. Add 2.3 µL of 10X NEBuffer 3.1 per reaction to the tube.

- The total 10X NEBuffer 3.1 added is **n × 2.3 µL**.

7.5.5. Add 2 µL of EcoRV (20,000 units/mL) endonuclease per reaction to the tube.

- The total EcoRV endonuclease added is **n × 2 µL**.
- Gently vortex to mix.

7.5.6. For each reaction, aliquot 20 µL of the above mix into individual wells in a PCR plate (or tube).
7.5.7. Add 10 µL of each FLT3 D835 amplicon to its corresponding wells containing the restriction digest mix.

- Pipette up and down several times to mix.

7.5.8. Seal PCR plate (or close tube) utilized for digestion step.
7.5.9. Incubate at 37°C for at least 60 minutes and up to 24 hours.

- Although amplified DNA is stable at room temperature for extended periods of time, store PCR products at 2°C to 8°C until detection.
- Detection must be within 30 days of amplification.

7.6. ABI Fluorescence Detection

Please note that for ABI fluorescence detection a preceding peak is often seen and is an artifact due to the detection method the ABI platforms use. Preceding peaks are sometimes skewed and have bases that slope on the right side towards the real peak.

Warning: Do not multiplex PCR products from different master mixes together as this will result in an overall reduced assay sensitivity.

7.6.1. In a new microcentrifuge tube, mix an appropriate amount (for a total of 10 µL per reaction) of Hi-Di Formamide with 600 LIZ Size Standards v2.0. Vortex well.
7.6.2. In a new 96-well PCR plate, add 10 µL of the above mixture of Hi-Di Formamide with 600 LIZ Size Standards v2.0 to individual wells for each reaction.
7.6.3. Transfer 0.5 µL of each amplicon to the wells containing Hi-Di Formamide with 600 LIZ size standards v2.0.

- Add only one sample per well.
- Pipette up and down to mix.

7.6.4. Seal the PCR plate with aluminum foil or 8-cap PCR strips.
7.6.5. Heat denature the samples at 95ºC for 3 minutes then snap chill on ice or at 4°C for 5 minutes.
7.6.6. Prepare a **sample sheet** and **injection list** for the samples.
7.6.7. Run the samples on an ABI capillary electrophoresis instrument according to the user manual.

- Data are automatically displayed as size and color specific peaks.

7.6.8. Review profile and controls, report results. (See sections 8*: Interpretation of Results* and 10.1: *Expected Size of Amplified Products* below.)

7.7. Quality Control

Positive and negative (or normal) controls are furnished with the kit and should be run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition, include a no template control (*e.g.,* water) to test for contamination. A buffer control may also be added to ensure that no contamination of the buffer used to resuspend the samples has occurred. The values for the positive controls are provided under section 10.1: *Expected Size of Amplified Products*.

8. Interpretation of Results

Interpret both positive and negative results in the context of all clinical information and laboratory test results. The size range for each master mix has been determined by testing positive and negative control samples. For accurate and meaningful interpretation, ignore peaks that occur outside of the valid size range for each of the master mixes.

- Note that there may be a +/- 4 bp variability in the size of a fragment inherent to the ABI instrument.
- Product lengths correspond to results obtained through the use of GeneScan - 600 LIZ Size Standard v2.0 and an ABI instrument. The use of different size standards and instruments may yield different product sizes.

8.1. Analysis

8.1.1. Report samples that fail to amplify following repeat testing as "A result cannot be reported on this specimen because there was DNA of insufficient quantity or quality for analysis".

8.1.2. The *FLT3* ITD Master Mix primers are labeled with FAM (blue) and HEX (green), forward and reverse primer, respectively. Wild-type (327 bp) and mutant peaks (any peak ≥ 330 bp) must contain both blue and a corresponding green peak to be considered a real peak.

8.1.3. The *FLT3* TKD Master Mix primers are labeled with FAM only. A positive *FLT3* TKD result consists of a mutant peak that is ≥ 1% of the WT peak.

- To calculate this value, divide the Mutant (124 bp or 127 bp for patient samples and 124 bp for the positive control) peak height by the WT (79 bp) peak height.
- A negative result will have a mutant peak that is < 1.0% of the WT peak.

8.1.4. All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples cannot be interpreted.

Table 6: The following describes the analysis of each of the controls, and the decisions necessary based upon the results.

Type of Control	Expected Result	Aberrant Result
***FLT3* ITD No Template Control**	No peaks ≥ 100 RFU and larger than 50 bp.	Amplification present, repeat the assay.
***FLT3* ITD Negative Control**	**Blue** and **Green** Peak at 327 bp ≥ 4,500 RFU	Insufficient amplification, positive peak/s detected; repeat the assay.
***FLT3* ITD Positive Control**	**Blue** and **Green** Peak at 327 bp ≥ 4,500 RFU and a **Blue** and **Green** peak at 357 bp ≥ 100 RFU	Insufficient amplification, mutant peak absent; repeat the assay.
***FLT3* TKD No Template Control**	No peaks ≥ 100 RFU and larger than 50 bp.	Amplification present, repeat the assay.
***FLT3* TKD Negative Control**	**Blue** Peak at 79 bp ≥ 4,500 RFU.	Insufficient amplification, positive peak/s detected; repeat the assay
***FLT3* D835 Positive Control**	**Blue** Peak at 79 bp ≥ 4,500 RFU and a **Blue** peak at 124 bp ≥ 1% of the WT peak.	Insufficient amplification, mutant peak < 1.0%; repeat the assay.
Specimen Control Size Ladder (Optional) This amplification control is suggested for samples of unknown quantity and quality.	If all 96, 197, 297, 397 and 602 bp peaks are observed, continue with analysis. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 bp fragment to have a diminished signal or to be missing entirely. Continue with analysis.	If no peaks are seen, repeat the assay unless specimen tests positive. If only 1, 2, or 3 peaks are seen, re-evaluate sample for DNA degradation unless specimen tests positive.

8.2. Sample Interpretation

Given that the controls generate expected results, interpret the clinical samples as follows:

Table 7: Sample interpretation

***FLT3* ITD Master Mix:**	
Positive:	Presence of mutant peaks (both FAM and HEX) ≥ 330 bp and ≥ 100 RFU is reported as: "**Detection of an ITD mutation of the *FLT3* gene**."
Negative:	Presence of the wild-type peak (both FAM and HEX) at 327 bp (≥ 4,500 RFU) with no peaks ≥ 330 bp and ≥ 100 RFU is reported as: "**No evidence of an ITD mutation of the *FLT3* gene**."
Failure to amplify:	Failure to produce a wild-type peak (327 bp for both FAM and HEX) of ≥ 4500 RFU with negative samples.
***FLT3* D835 Master Mix:**	
Positive:	Presence of the 124 bp or 127 bp mutant peak (FAM) that is ≥ 1.0% of the 79 bp wild-type peak is reported as: "**Detection of a TKD mutation of the *FLT3* gene**."
Negative:	Presence of the wild-type peak (FAM) at 79 bp (≥ 4500 RFU), with a < 1.0% of the mutant present is reported as: "**No evidence of a TKD mutation of the *FLT3* gene**."
Failure to amplify:	Failure to produce a wild-type peak (FAM) of ≥ 4500 RFU with negative samples.

9. Limitations of Procedure

- This assay does not identify 100% of *FLT3* activating mutations.
- This assay cannot reliably detect less than five (5) positive cells per 100 normal cells.
- Always interpret the results of molecular tests in the context of clinical, histological and immunophenotypic data.
- PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA, heparin, and other agents.

10. Sample Data

10.1. Expected Size of Amplified Products

The amplicon sizes listed were determined using an ABI 3500xL platform. Amplicon sizes seen on each specific capillary electrophoresis instrument may differ 1 to 4 base pair (bp) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on each specific platform will be consistent from run to run. This reproducibility is extremely useful when monitoring disease recurrence.

Note: "Color" indicates the color of products generated with the master mix when using the default color assignment on ABI fluorescence detection systems.

Table 8: Expected Size of Amplified Products for *FLT3* ITD Master Mix

Master Mix	Target	Color	Sample	Product Size in bp
***FLT3* ITD**	**ITD**	**Blue** & **Green**	No Template Control	No peaks > 100 RFU and larger than 50 bp.
		Blue & **Green**	*FLT3* Negative Control DNA (REF 40920030)	Blue and Green Peak at 327 bp ≥ 4,500 RFU
		Blue & **Green**	*FLT3* ITD Positive Control (REF 40883390)	Blue and Green Peak at 327 bp ≥ 4,500 RFU and a Blue and Green peak at 357 bp ≥ 100 RFU
		Blue & **Green**	*FLT3* ITD Negative Patient Sample	Blue and Green Peak at 327 bp ≥ 4,500 RFU
		Blue & **Green**	*FLT3* ITD Positive Patient Samples	Blue and Green Peak at ≥ 330 bp ≥ 100 RFU. **Note:** The WT peak at 327 bp may or may not be present.

Table 9: Expected Size of Amplified Products for FLT3 TKD Master Mix

Master Mix	Target	Color	Sample	Product Size in bp
***FLT3* D835**	**TKD**	**Blue**	No Template Control	No peaks ≥ 100 RFU and larger than 50 bp.
		Blue	*FLT3* Negative Control DNA (REF 40920030)	Blue Peak at 79 bp ≥ 4,500 RFU
		Blue	*FLT3* D835 Positive Control (REF 40883400)	Blue Peak at 79 bp ≥ 4,500 RFU and a Blue peak at 124 bp at least 1% of the WT peak
		Blue	*FLT3* TKD Negative Patient Sample	Blue Peak at 79 bp ≥ 4,500 RFU
		Blue	*FLT3* TKD Positive Patient Samples	Blue Peak at 124 bp or 127 bp that is at least 1% of the WT peak. **Note:** The WT peak at 79 bp may or may not be present.

Note on the TKD interpretation: Some EcoRV undigested product might be present and detectable as a blue peak at 147 bp. If only the 147 bp peak is detected or the 147 bp peak is ≥ 1% of the WT peak, the sample must be repeated by either starting at the amplification step or the EcoRV digestion step.

10.2. Sample Data

The data shown below was generated using kit provided master mixes and controls with product detection on an ABI platform.

Note: A 1-4 base pair (bp) difference in size detected is inherent to intra-instrument variance across capillary detection platforms.

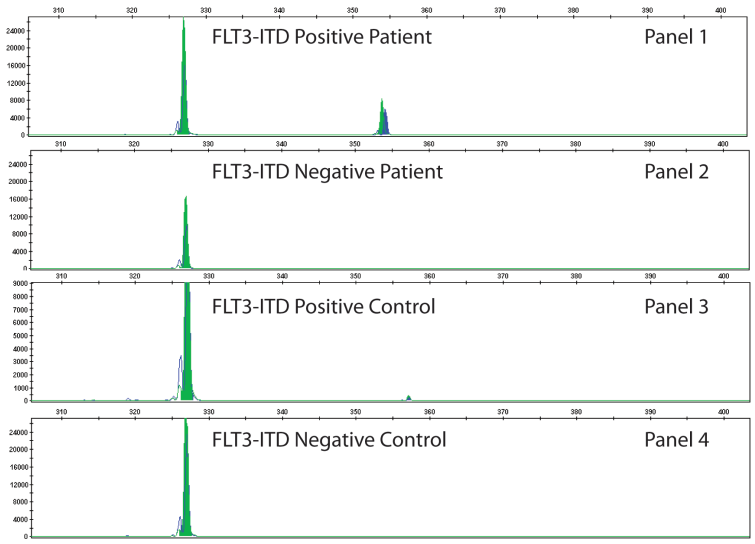

Figure 2. *FLT3* ITD Master Mix. Data panels demonstrate capillary peak detection for FLT3-ITD Positive Patient (panel 1), FLT3-ITD Negative Patient (panel 2), FLT3-ITD Positive Control kit component REF 40883390 (panel 3), and FLT3-ITD Negative Control kit component REF 40920030 panel 4).

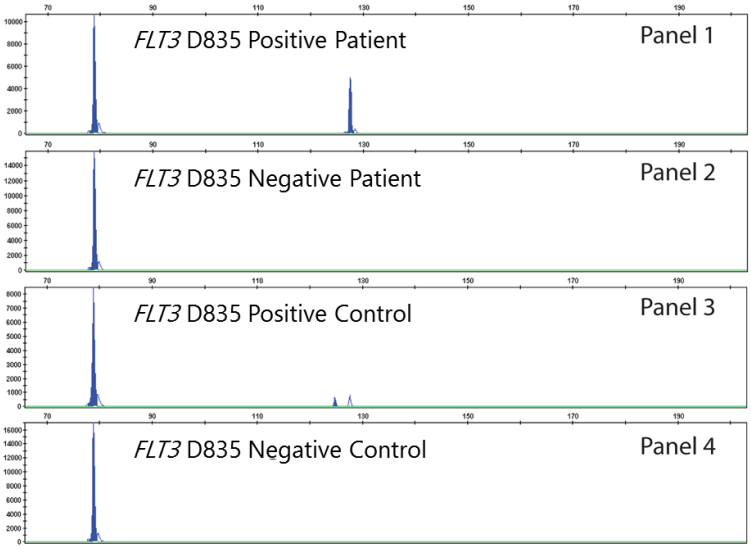

Figure 3. *FLT3* D835 Master Mix. Data panels demonstrate capillary peak detection for *FLT3*-D835 Positive Patient (panel 1), *FLT3*-D835 Negative Patient (panel 2), *FLT3*-D835 Positive Control kit component REF 40883400 (panel 3), and *FLT3*-D835 Negative Control kit component REF 40920030 panel 4).

11. Performance Characteristics

The LeukoStrat *FLT3* Mutation Assay 2.0 validation provides evidence that the assay is capable of detecting *FLT3* ITD and TKD mutations with a concordance of ≥ 90% when compared to Roche®454 sequencing. The validation study challenged the assay's ability to identify *FLT3* mutations while assessing the impact of multiple operators, reagent lots, ABI 3500xL instruments, and nonconsecutive testing days. The PPA and NPA for clinical samples, contrived positive samples, and contrived negative samples exceeded 90% between the LeukoStrat *FLT3* Mutation Assay 2.0 and 454 sequencing results.

11.1. Data Summary

The *FLT3* ITD contrived samples consisted of a 5% Positive ITD, 50% Positive ITD, and Negative ITD. Each contrived sample was tested in replicates of three using two different master mix lots, two different operators, over the course of 5 non-consecutive days, producing a total of 60 replicates for each contrived sample. A total of 10 clinical samples were tested in singlicate, using two different master mix lots, with two different operators, over the course of five (5) non-consecutive days, producing a total of 20 replicates of each clinical sample. Per protocol, runs with failed controls were not included in the final analyses. A 100% and 98% agreement was achieved for the contrived and clinical samples, respectively (refer to Table 11: ITD Sample Size and Sample Agreement). The Positive and Negative controls were not included in the calculations. Clinical sample ITD-6 was misclassified in four (4) of the 20 replicates.

Table 10: ITD Sample Size and Sample Agreement with 454 Sequencing

Sample	Result	N	% Agreement	Comments
Pos. Control	Positive	17	100%	N/A
5% Positive	Positive	51	100%	N/A
50% Positive	Positive	51	100%	N/A
ITD-1	Positive	17	100%	N/A
ITD-3	Positive	17	100%	N/A
ITD-4	Positive	17	100%	N/A
ITD-5	Positive	17	100%	N/A
ITD-6	Negative	4	20%	FAM (HEX peaks 101 – 113 RFU)
	Positive	13	80%	
ITD-7	Positive	17	100%	N/A
Neg. Control	Negative	17	100%	N/A
Negative	Negative	51	100%	N/A
ITD-2	Negative	17	100%	N/A
ITD-8	Negative	17	100%	N/A
ITD-9	Negative	17	100%	N/A
ITD-10	Negative	17	100%	N/A

The *FLT3* TKD contrived samples consisted of a 5% Positive TKD, 50% Positive TKD, and Negative TKD. Each contrived sample was tested in replicates of three using two different master mix lots, two different operators, over the course of five (5) non-consecutive days, producing a total of 60 replicates for each contrived sample. A total of 10 clinical samples were tested in singlicate, using two different master mix lots, with two different operators, over the course of five (5) non-consecutive days, producing a total of 20 replicates for each clinical sample. A 100% agreement was achieved for contrived and clinical samples (refer to Table 12: TKD Sample Size and Sample Agreement). The Positive and Negative controls were not included in the calculations. The Negative TKD contrived sample had three of 60 replicates that failed to amplify sufficiently and therefore were excluded from the NPA analysis set.

Table 11: TKD Sample Size and Sample Agreement with 454 Sequencing

Sample	Result	N	% Agreement	Comments (discrepancies)
Pos. Control	Positive	20	100%	N/A
5% Positive	Positive	60	100%	N/A
50% Positive	Positive	60	100%	N/A
TKD-2	Positive	20	100%	N/A
TKD-3	Positive	20	100%	N/A
TKD-4	Positive	20	100%	N/A
TKD-6	Positive	20	100%	N/A
TKD-7	Positive	20	100%	N/A
TKD-10	Positive	20	100%	N/A
Neg. Control	Negative	20	100%	N/A
Negative	Negative	57	100%	3/60 (5%) did not amplify sufficiently
TKD-1	Negative	20	100%	N/A
TKD-5	Negative	20	100%	N/A
TKD-8	Negative	20	100%	N/A
TKD-9	Negative	20	100%	N/A

11.2. Data Analysis

For *FLT3* ITD known negative samples there were zero (0) discordant results obtained (119/119 concordance), producing a NPA of 100% (refer to Table 13: ITD Percent Agreement with 454 Sequencing).

For *FLT3* ITD known positive samples there were four (4) discordant results obtained (200/204 concordance), producing a PPA of 98.0% (refer to Table 13: ITD Percent Agreement with 454 Sequencing). Clinical sample ITD-6 was a known positive for *FLT3* ITD per 454 sequencing, however four of the 20 replicates tested using the LeukoStrat *FLT3* Mutation Assay 2.0 returned negative results.

Table 12: ITD Percent Agreement with 454 Sequencing

Percent Agreement		# Discordant	# Concordant	*95% LL
NPA	100%	0	119	96.9%
PPA	98.0%	4	200	95.1%

*95% of results would be expected to agree with sequencing at a rate greater than or equal to the lower limit (LL).

For the *FLT3* TKD known negative samples, there were zero (0) discordant results obtained (137/137), producing a NPA of 100% (refer to Table 14: TKD Percent Agreement with 454 Sequencing) between the 454 results and the LeukoStrat *FLT3* Mutation Assay 2.0.

For the *FLT3* TKD known positive samples, there were zero (0) discordant results obtained (240/240), producing a PPA of 100% (refer to Table14: TKD Percent Agreement with 454 Sequencing) between the 454 results and the LeukoStrat *FLT3* Mutation Assay 2.0.

Table 13: TKD Percent Agreement with 454 Sequencing

Percent Agreement		Discordance #	Concordance #	*95% LL
NPA	100%	0	137	96.9%
PPA	100%	0	240	98.5%

*95% of results would be expected to agree with sequencing at a rate greater than or equal to the lower limit (LL).

The overall run acceptability rate for *FLT3* ITD and *FLT3* TKD was 85% and 100%, respectively. The individual sample validity rates for ITD and TKD were 85% and 99.2% respectively.

11.3. Conclusion

This validation provides documented evidence that the LeukoStrat *FLT3* Mutation Assay 2.0 is capable of detecting *FLT3* ITD and *FLT3* TKD mutations with a minimum concordance of 90% when compared to 454 Sequencing.

12. References

1. Murphy, KM. et al., (2003). Detection of FLT3 Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Chain Reaction and Capillary Electrophoresis Assay. The Journal of Molecular Diagnostics 5, 96 – 102.
2. Yamamoto, Y. et al., (2001). Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 97, 2434-2439.

13. Technical and Customer Service

Thank you for purchasing our **LeukoStrat *FLT3* Mutation Assay 2.0 – ABI Fluorescence Detection**. We appreciate your business. We are happy to assist you in the validation of this assay and will provide ongoing technical assistance to keep the assays performing efficiently in your laboratory. Technical assistance is most rapidly obtained using our Internet site: **http://www.invivoscribe.com** or by sending an email inquiry to: **support@invivoscribe.com.**

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14. Symbols

The following symbols are used in Invivoscribe diagnostic product labeling.

REF	Catalog Number		Expiration Date
VOL	Reagent Volume	EC REP	Authorized Representative in the European Community
LOT	Lot Number		Consult Instructions for Use
	Storage Conditions	IVD	For *In Vitro* Diagnostic Use
UDI	Unique Device Identifier		Manufacturer
UK CA	UK Conformity Assessed	UKRP	UK Responsible Person
CH REP	Swiss Authorized Representative	CE	European Conformity

15. Legal Notice

This product is an *in vitro* diagnostic product; not available for sale or use within North America.

15.1. Warranty and Liability

Invivoscribe, Inc. (Invivoscribe®) is committed to providing the highest quality products. Invivoscribe® warrants that the products meet or exceed the performance standards described in the Instructions For Use, as to products with such an insert. If a product is covered by product specifications and does not perform as specified, our policy is to replace the product or credit the full purchase price. No other warranties of any kind, expressed or implied, are provided by Invivoscribe®. Invivoscribe® liability shall not exceed the purchase price of the product. Invivoscribe shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products; product efficacy under purchaser controlled conditions in purchaser's laboratory must be established and continually monitored through purchaser defined and controlled processes including but not limited to testing of internally validated positive, negative, and blank controls every time a sample is tested. Ordering, acceptance and use of product constitutes purchaser acceptance of sole responsibility for assuring product efficacy and purchaser agreement to the limitation of liability set forth in this paragraph.

15.2. Patents and Trademarks

Many of these products require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). No license under these patents to use amplification processes or enzymes is conveyed expressly or by implication to the purchaser by the purchase of this product.