LeukoStrat®

Instructions for Use

LeukoStrat® *FLT3* Mutation Assay

CE IVD

For Identification of *FLT3* ITD and TKD mutations.

IVD For *In Vitro* Diagnostic Use

invivoscribe®

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

Catalog #		Products	Quantity
REF	94120010	LeukoStrat *FLT3* Mutation Assay – Gel Detection	33 Reactions
REF	94120020	LeukoStrat *FLT3* Mutation Assay MegaKit – Gel Detection	330 reactions

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1. Intended Use

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

This assay cannot reliably detect *FLT3* mutations comprising less than 5% of the total cell population. **Always interpret the results of molecular mutation tests in the context of clinical, histological and immunophenotypic data**.

2.2. Summary

This test kit includes three (3) master mixes and two (2) controls. The *FLT3* ITD master mix tests for internal tandem duplication mutations, and the *FLT3* D835 master mix tests for TKD region mutations. The Specimen Control Size Ladder master mix targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400, and 600 base pairs (bp) to ensure that the quality and quantity of input DNA is adequate to yield a valid result. Kit components include controls for ITD and TKD mutations in the *FLT3* gene.

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 genetic analyzer, wild-type *FLT3* alleles will amplify and produce a 327±1 bp product using this assay, while alleles that contain ITD mutations will produce a product that exceeds 327±1 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, and when a nucleic acid substitution occurs, the restriction digest recognition site disappears, rendering the EcoRV endonuclease unable to identify and digest the DNA at this site. The LeukoStrat *FLT3* Mutation Assay uses primers that lie on either side of the TKD region, in which the *FLT3* target region is amplified using PCR followed by EcoRV restriction digest. Since one of the primers contains an EcoRV restriction site, both wild type and mutant alleles are digested creating a digestion pattern that identifies loss of the normal gene sequence and ensures that digestion occurred. When interrogating human gDNA template on an ABI 3500xL genetic analyzer, 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).

3.3. Gel Detection

Gel electrophoresis, such as agarose gel electrophoresis or non-denaturing polyacrylamide gel electrophoresis (PAGE), is commonly used to resolve the different amplicon products based on their size, charge, and conformation. Since DNA is negatively charged, when an electrical potential (voltage) is applied across the gel containing PCR products, the electrical field causes the amplicons to migrate through the gel. Smaller DNA fragments are able to easily migrate through the gel matrix, whereas larger

DNA fragments migrate more slowly which causes a separation of the amplicon products based on size. Ethidium bromide or other DNA intercalating dyes can then be used to stain and detect these products in the gel.

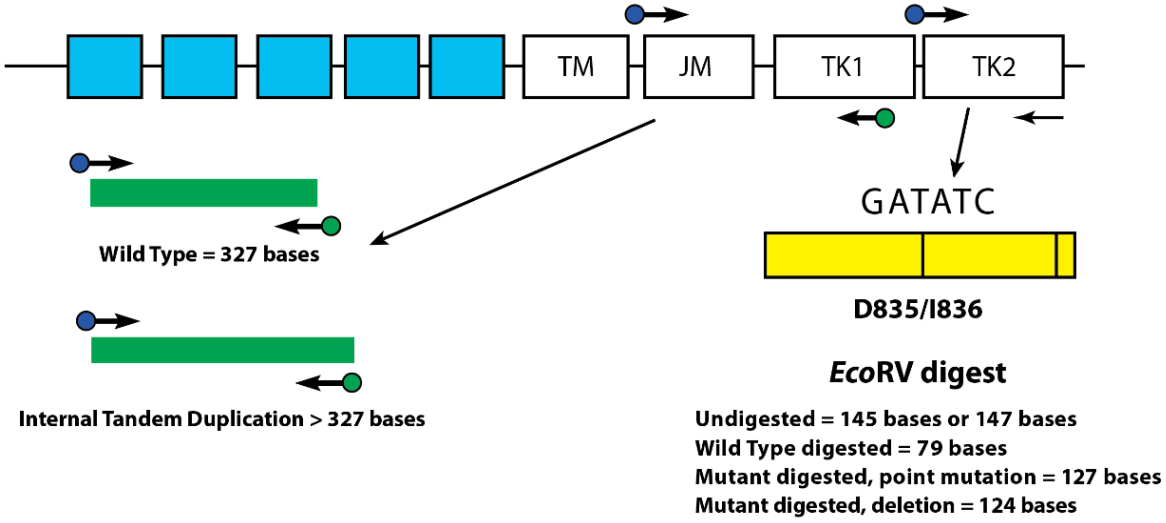

Figure 1. Depicted is a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target in and around the JM region for ITD or the activating loop of the kinase domain for TKD. Colored dots represent fluorophores on labeled primers. The yellow box has vertical black lines that represent the position of the EcoRV restriction digest sites. **Product sizes reflect human gDNA measured on an ABI 3500xL Genetic Analyzer.**

4. Reagents

4.1. Reagent Components

Table 1: Available Kits

Catalog #	Description	Quantity
REF 94120010	LeukoStrat *FLT3* Mutation Assay – Gel Detection	33 Reactions
REF 94120020	LeukoStrat *FLT3* Mutation Assay MegaKit – Gel Detection	330 Reactions

Table 2: Kit Components

Reagent	Catalog # (REF)	Reagent Components (active ingredients)	Unit Quantity	94120010 # of Units	94120020 # of Units	Storage Temp.
Master Mixes	24120010CE	***FLT3* ITD Master Mix – Unlabeled** Multiple oligonucleotides targeting the *FLT3* gene in a buffered salt solution.	1500 µL	1	10	-85°C -65°C
	24120020CE	***FLT3* D835 Master Mix – Unlabeled** Multiple oligonucleotides targeting the *FLT3* TKD region in a buffered salt solution.	1500 µL	1	10	
Template Amplification Control Master Mix	20960020	**Specimen Control Size Ladder – Unlabeled** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	
Positive Control DNA	40882960	**IVS-0050 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	2°C 8°C

Table 2: Kit Components

Reagent	Catalog # (REF)	Reagent Components (active ingredients)	Unit Quantity	94120010 # of Units	94120020 # of Units	Storage Temp.
	40900180	**IVS-P004 Clonal Control DNA** 171 pg/mL of plasmid DNA diluted in IVS-0000 Polyclonal Control DNA in 1/10th TE solution	100 µL	1	5	or -65°C -85°C
Negative (Normal) Control DNA	40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	8°C 2°C or -65°C -85°C

Note: There are no preservatives used in the manufacture of this kit.

4.2. Warnings and Precautions

- 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. Use care to ensure use of correct thermal cycler program, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
- Do not mix or combine reagents from kits with different lot numbers.
- Wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets. Use molecular biology grade water for the preparation of specimen DNA.
- Due to the analytical sensitivity of this test, use extreme care to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. Closely monitor all 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.
- Autoclaving does not eliminate DNA contamination. Follow uni-directional workflow in the PCR laboratory; begin with master mix preparation, move to specimen preparation, then to amplification, and finally to detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
- Dedicate all pipettes, pipette tips, and any equipment used in a particular area to that area of the laboratory.
- Use sterile, disposable plastic ware whenever possible to avoid RNase, DNase, or cross-contamination.

4.3. Storage and Handling

- For any duration other than immediate use, s**tore 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 long term at -85°C to -65°C.
- 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.
- 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
- 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. Electrophoresis Unit

- Use or function: DNA fragment separation
- Performance characteristics and specification:
  - Capable of running at 35V to 135V for extended times
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.

5.3. UV Illumination Unit

- Use or function: DNA detection
- Performance characteristics and specification:
  - Capable of emitting light at a wavelength of ~302 nm
- Follow manufacturer's installation, operation, calibration, and maintenance procedures.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle all specimens in accordance with the OSHA Standard on Blood borne Pathogens 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

6.3. Specimen Requirements and Handling

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

- 5 cc of peripheral blood, bone marrow biopsy, or bone marrow aspirate anti-coagulated with heparin or EDTA (stored at 2°C to 8°C and shipped at ambient temperature)
- Minimum 5 mm cube of tissue (stored and shipped frozen; or stored and shipped in RPMI 1640 at ambient temperature or on ice)
- 2 µg of gDNA (stored at 2°C to 8°C and shipped at ambient temperature)
- Formalin-fixed paraffin embedded tissue or slides (stored and shipped at ambient temperature)

6.4. Sample Preparation

Extract the gDNA from patient specimens as soon as possible. Resuspend DNA to a final concentration of 100 µg to 400 µg per mL in $1/10^{th}$ TE (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) or in molecular biology grade or USP water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNA with the Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

6.5. Sample Storage

Store gDNA at 2°C to 8°C or for long term at -85°C to -65°C.

7. Assay Procedure

7.1. Materials Provided

Table 3: Kit components

Catalog #	Description
REF 24120010CE	*FLT3* ITD Master Mix – Unlabeled
REF 24120020CE	*FLT3* D835 Master Mix – Unlabeled
REF 20960020	Specimen Control Size Ladder – Unlabeled
REF 40882960	IVS-0050 Clonal Control DNA
REF 40900180	IVS-P004 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

7.2. Materials Required (not provided)

Table 4: Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog # (REF)	Notes
DNA Polymerase	Roche: • EagleTaq DNA Polymerase Invivoscribe, Inc.: • EagleTaq DNA Polymerase[1] or equivalent	05206944190 60970100	N/A
EcoRV endonuclease	New England BioLabs: • EcoRV 20,000 units/mL	R0195L	N/A
EcoRV Buffer	New England BioLabs: • 10X NEBuffer 3	B7003s	Typically included with the EcoRV restriction enzyme
Molecular Biology Grade or USP Water	N/A	N/A	Sterile and free of DNase and RNase.
Calibrated Pipettes	Rainin: • P-2, P-20, P-200, and P-1000 pipettes • Or SL-2, SL-20, SL-200, and SL-1000 pipettes	N/A	Must be able to accurately measure volumes between 1 µL and 1000 µL.
Thermal cycler	Bio-Rad: • MJ Research PTC-100 or PTC-200, PTC-220, PTC-240 Perkin-Elmer • PE 9600 or PE 9700	N/A	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
Gel Electrophoresis Unit	N/A	N/A	For polyacrylamide gels
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure™ 10 mg/mL Ethidium Bromide	15585-011	N/A
Agarose Gel	Thermo Fisher Scientific: • NuSieve® 3:1 Agarose	50091	N/A
TBE Running Buffer	Thermo Fisher Scientific: • Novex TBE Running Buffer (5X)	LC6675	Dilute 1:5 prior to use.

Table 4: Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Catalog # (REF)	Notes
Gel Loading Buffer	Thermo Fisher Scientific: • 10X BlueJuice™ Gel Loading Buffer	10816-015	N/A
100 bp DNA Ladder	Thermo Fisher Scientific: • TrackIt™ 100 bp DNA Ladder	10488-058	N/A

[1]**Note:** This product is for sale and use in the European Economic Area only. It is not to be resold or transferred to another party. See also Legal Notice in section 15.

7.3. Reagent Preparation

- 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.
- Singlicate test results are valid; however, test in **duplicate** when possible. If duplicate testing provides inconsistent results, re-testing or re-evaluation of the sample may be necessary.
- Test **positive**, **negative** and **no template** controls with each master mix.

7.3.1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw completely; then gently vortex to mix.

7.3.2. 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 correct for pipeting errors.
- Thus, for each master mix (except for the Specimen Control Size Ladder), the number of reactions (**n**) is:

n = 2 × # of samples	(run each sample in duplicate)
+ 1	positive control DNA (See section *7.7 Recommended Positive Controls*)
+ 1	negative control DNA (IVS-0000 Polyclonal Control DNA)
+ 1	no template control (water)
+ 1	to correct for pipetting errors
n = 2 × # of samples + 4	**Total**

- Therefore the total aliquot volume for each master mix is n × 45 µL.
- For the Specimen Control Size Ladder master mix, the number of reactions (**m**) is:

m = # of samples	(run each sample in singlicate)
+ 1	positive control DNA (IVS-0000 Polyclonal Control DNA)
+ 1	no template control (water)
+ 1	to correct for pipetting errors
m = # of samples + 3	**Total**

- Therefore the total aliquot volume for the Specimen Control Size Ladder master mix is **m × 45 µL**.

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

- The total EagleTaq DNA polymerase added to each master mix is **n × 0.25 µL**, and **m × 0.25 µL** for the Specimen Control Size Ladder master mix.
- Gently vortex to mix.

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

7.3.5. Add 5 µL of appropriate template (sample DNA, positive control DNA, negative control DNA, or water) to the individual wells containing the respective master mix solutions.

- Pipette up and down several times to mix.

7.3.6. Cap or cover the PCR plate.

- Samples are now ready to be amplified on a thermal cycler.
- If amplification cannot be performed immediately following reagent preparation, the PCR plate or tubes can be stored at 2°C to 8°C for up to 24 hours.

Quick Guide:

For each master mix and **n** reactions, mix:

n × 45 µL Master Mix
n × 0.25 µL Taq DNA polymerase
Vortex gently to mix.
Aliquot **45 µL** of master mix + DNA polymerase solution into each reaction well.
Add **5 µL** of appropriate Template to each well
Total reaction volume = **50 µL**

7.4. Amplification

7.4.1. Amplify the samples using the following PCR program:

- Use the **calculated** option for temperature measurement with the BioRad MJ Research PTC thermal cyclers.

Table 5: Thermal cycling conditions

Step	Temperature	Duration	Cycles
1	95°C	7 minutes	1
2	94°C	30 seconds	35
3	55°C	30 seconds	
4	72°C	60 seconds	
5	72°C	10 minutes	1
6	15°C	∞	1

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

- 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.

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

7.5.1. Using gloved hands, remove the 10X NEBuffer 3 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.

- Include an additional reaction for every 15 reactions to correct for pipeting errors.
- Thus, the number of reactions (**n**) is:

n = 2 × # of samples	(run each sample in duplicate)
+ 1	to correct for pipetting errors
n = 2 × # of samples + 1	**Total**

7.5.3. Add 16 µL of molecular grade water per reaction to each tube.

- The total water added is **n × 16 µL**.

7.5.4. Add 2 µL of 10X NEBuffer3 per reaction to each tube.

- The total 10X NEBuffer3 added is **n × 2 µL**.

7.5.5. Add 40 units (or 2 µL at 20,000 U/mL) of EcoRV endonuclease per reaction to each 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 solution into individual wells in a PCR plate or tube.

7.5.7. Add 10 µL of each *FLT3* D835 Master Mix amplified sample/control to the individual wells containing the solution above.

- Pipette up and down several times to mix.

7.5.8. Cap or cover the PCR plate.

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. Gel Detection – Agarose Gel

7.6.1. Assemble the electrophoresis unit using a 2% NuSieve 3:1 Agarose TBE gel containing 0.4 µg/mL of Ethidium Bromide and 1X TBE running buffer.

7.6.2. Mix 20 µL of each sample with 4 µL of 6X loading buffer.

7.6.3. Load 20 µL of the mixture into individual wells of the gel.

7.6.4. Run gel at 100V for 55 minutes.

7.6.5. Place gel over UV illuminator to visualize bands

7.6.6. Photograph and interpret resulting data. (See sections 8: *Interpretation of Results* and 10: *Expected Values*)

7.7. Interpretation of ResultsQuality Control

Positive and negative (or normal) controls are furnished with the kit and can 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 of the master mix or cross-contamination of reactions. 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: *Reference Sample Data*.

Table 6: Product sizes as determined using kit provided Master Mixes/Controls with capillary electrophoresis detection on an ABI 3100 platform. A 1-4 base pair (bp) difference intra-instrument may be expected.

Master Mix	Target	Control DNA	Catalog #	Product Size in base pairs (bp)
***FLT3* ITD Master Mix**	ITD	**Valid Size Range** IVS-0050 Clonal Control DNA	--- 40882960	**330 - 1000** 360
***FLT3* D835 Master Mix**	TKD	**Valid Size Range** IVS-P004 Clonal Control DNA	--- 40900180	**150 - undigested** 150 – undigested 129 - digested
Specimen Control Size Ladder	Multiple Genes	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 40920010	**100, 200, 300, 400, 600[a]** 100, 200, 300, 400, 600[a]

[a]**Note**: 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.

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 bands that occur outside of the valid size range for each master mix.

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. Repeat testing on samples that test negative if the positive control reaction failed.

8.1.3. If samples run in duplicate yield differing results, re-test and/or re-evaluate samples for sample switching.

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 7. 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
No Template Control	No amplification present, continue with analysis	Amplification present, repeat the assay.
Polyclonal Control	Product size is consistent with expected size listed in section 10: *Expected Values*. Continue with Analysis.	Positive bands listed in section 10.1: *Reference Sample Data* are present. Repeat the assay
Positive Control (This can also be an extraction control if positive control material is taken through extraction processes)	Product size is consistent with expected size listed in section 10: *Expected Values*. Continue with Analysis.	Repeat the assay.
Specimen Control Size Ladder (This amplification control is essential for samples of unknown quantity and quality.)	If all of the 100, 200, 300, 400, and 600 bp peaks are seen, 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 bands are seen, repeat the assay unless specimen tests positive. If only 1, 2, or 3 bands are seen, re-evaluate sample for DNA degradation unless specimen tests positive.

8.2. Sample Interpretation

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

8.2.1. *FLT3* ITD Master Mix:

- **Positive:** Presence of band(s) larger than 335 bp is reported as: "**Detection of an ITD mutation of the *FLT3* gene**."
- **Negative:** Presence of a band at ~330 bp with no bands larger than 335 bp is reported as: "**No evidence of an ITD mutation of the *FLT3* gene**."

8.2.2. *FLT3* D835 Master Mix:

- **Positive:** Presence of a band at ~150 bp (undigested) and ~130 bp with no band at ~150 bp (EcoRV digested) is reported as: "**Detection of a TKD mutation of the *FLT3* gene**."
- **Negative:** Presence of a band at ~150 bp (undigested) and at ~81 bp (EcoRV digested) with no band at ~130 bp or ~150 bp (EcoRV digested) is reported as: "**No evidence of a TKD mutation of the *FLT3* gene**."

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. Expected Values

10.1. Reference Sample Data

Table 8: As a reference, the product sizes were determined using kit provided Master Mixes/Controls with capillary electrophoresis detection on an ABI 3100 platform. A 1-4 base pair (bp) difference intra-instrument may be expected.

Master Mix	Target	Control DNA	Catalog #	Product Size in base pairs (bp)
***FLT3* ITD Master Mix**	ITD	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0050 Clonal Control DNA	--- 40920010 40882960	**330 - 1000** 330 360
***FLT3* D835 Master Mix**	TKD	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-P004 Clonal Control DNA	--- 40920010 40900180	**150 - undigested** 150 – undigested 80 – digested 150 – undigested 129 - digested
Specimen Control Size Ladder	Multiple Genes	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 40920010	**100, 200, 300, 400, 600[a]** 100, 200, 300, 400, 600[a]

[a]**Note:** 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.

10.2. Sample Data

The data shown below were generated using the master mixes and controls indicated with amplified products run on a 2% agarose gel.

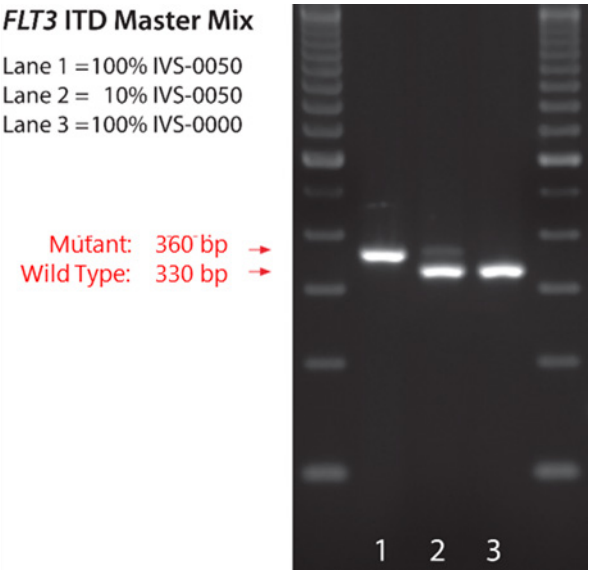

Figure 2. *FLT3* ITD Master Mix.

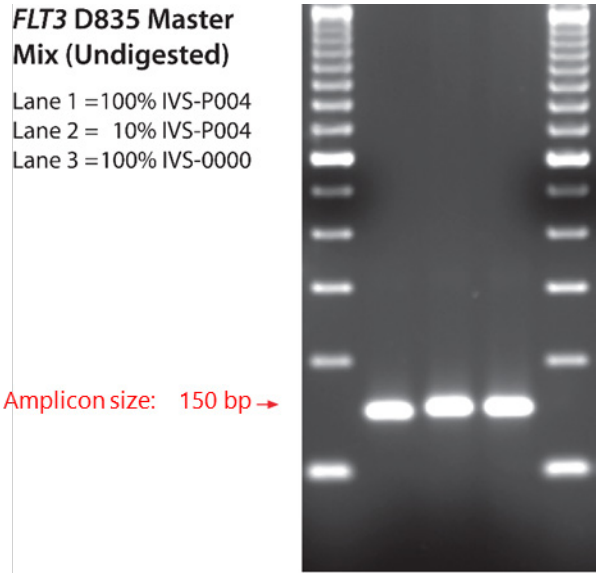

Figure 3. *FLT3* D835 Master Mix (undigested).

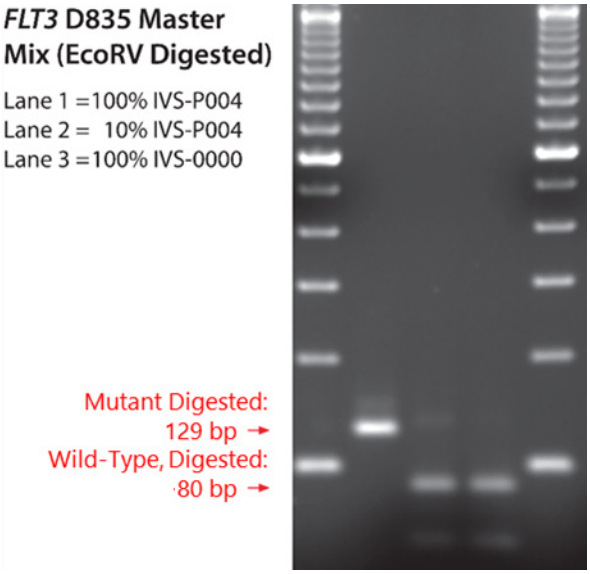

Figure 4. *FLT3* D835 Master Mix (EcoRV digested).

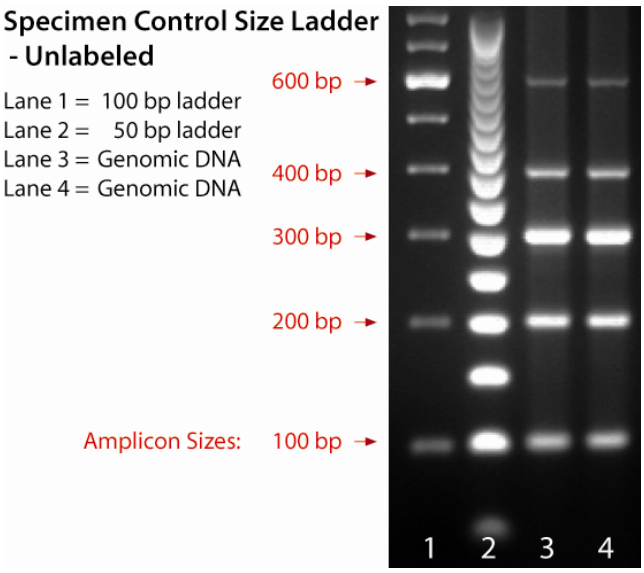

Figure 5. Specimen Control Size Ladder Master Mix.

11. Performance Characteristics

This LeukoStrat *FLT3* Mutation Assay is a rapid and reliable procedure in detecting *FLT3* mutations. This is evidenced by a validation performed by the Laboratory for Personalized Molecular Medicine (LabPMM).

Table 9. Concordance study results

Target	Concordance with three independent labs:
ITD	100% sensitivity/ 100% specificity
TKD	100% sensitivity/ 100% specificity

LabPMM tested fifty-seven (57) blinded patient samples obtained from three independent institutions. Thirteen (13) had been determined to be *FLT3* ITD positive and thirty-three (33) had been determined to be *FLT3* ITD negative by the independent institutions. Six (6) had been determined as *FLT3* TKD positive and fifty (50) *FLT3* TKD negative by the institutions that had provided the samples. In addition, ten (10) positive blinded, spiked samples and ten (10) negative samples were used for the validation of *FLT3* TKD. The LeukoStrat *FLT3* Mutation Assay showed a sensitivity and specificity of 100% with both master mixes. The analytical sensitivity of both master mixes was determined to be five (5) positive cells in 100 total cells.

12. Technical and Customer Service

Technical and Customer Service Representatives are available Monday through Friday to answer phone, e-mail, or website inquiries.

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13. Bibliography

1. Murphy KM, Smith BD, Levis M, Small D and Berg KD. A Clinical PCR/Capillary Electrophoresis Assay for the Detection of Internal Tandem Duplication and Point Mutation of the *FLT3* Gene. *Journal of Molecular Diagnostics.* 2003, **5:**96-102.
2. Yamamoto, Y., *et al.,* Activating mutation of D835 within the activation loop of *FLT3* in human hematologic malignancies. *Blood*. 2001, **97**(8):2434-9.

14. Symbols

The following symbols are used in Invivoscribe product labeling.

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

15. Legal Notice

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 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.

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

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.

LeukoStrat® is a registered trademark of Invivoscribe®.

15.3. Notice to Purchaser – EagleTaq DNA Polymerase ONLY

This product is for sale for research use in the European Economic Area (EEA) only. It is not to be resold or transferred to another party. Use of this product is covered by US Patent No. 6,127,155 and corresponding patent claims outside the US. This purchaser of this product may use this amount of product only for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Human and veterinary diagnostic uses under Roche patent claims require a separate license from Roche. All uses other than internal research and human and veterinary diagnostic uses under Roche patent claims require a separate license from Thermo Fisher Scientific. By using this product, you acknowledge your agreement to the above. Further information on purchasing licenses from Roche may be obtained by contacting the Licensing Department of Roche Molecular Systems, Inc., 4300 Hacienda Drive, Pleasanton, California 94588, USA or Roche Diagnostics GmbH, Sandhofer Strasse 116, 68305 Mannheim, Germany. Further information on purchasing licenses from Thermo Fisher Scientific may be obtained by contacting the Licensing Department of Thermo Fisher Scientific, 5791 Van Allen Way, Carlsbad, California 92008, USA.