LeukoStrat

Instructions for Use

LeukoStrat® CDx *FLT3* Mutation Assay

CE 2797 IVD

For detection of internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations in the FMS-like tyrosine kinase 3 (*FLT3*) gene.

IVD   For *In Vitro* Diagnostic Use

✳ invivoscribe®

Catalog #	Product	Quantity
REF K4120431	LeukoStrat CDx *FLT3* Mutation Assay	33 Reactions

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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 duplication (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 may be used as a companion diagnostic for the following therapeutic:

In regions where XOSPATA® (gilteritinib fumarate) is available, the LeukoStrat CDx *FLT3* Mutation Assay is used as an aid in the assessment of patients with AML for whom XOSPATA (gilteritinib fumarate) treatment is being considered.

In regions where VANFLYTA® (quizartinib hydrochloride) is available, the LeukoStrat CDx *FLT3* Mutation Assay is used as an aid in the assessment of patients with *FLT3*-ITD+ AML for whom VANFLYTA® (quizartinib hydrochloride) treatment is being considered.

The qualitative, non-automated test is for use on the 3500xL or 3500xL Dx Genetic Analyzers.

3.   Indications/Contraindications

There are no identified contraindications.

4.   Glossary

LeukoStrat CDx *FLT3* Software	LeukoStrat CDx *FLT3* Mutation Assay data analysis software.
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.
EC	Extraction Control
NTC	No Template Control
PC	Positive Control
Signal Ratio (SR)	Calculated by dividing the mutant peak area by the wild-type peak area.
Tyrosine Kinase Domain (TKD) Mutation	Nucleotide change(s) that result 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 *FLT3* gene.

5.   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 making it an attractive target for treatment.[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 and assay specific software to determine if *FLT3* mutations are present in human DNA extracted from mononuclear cells isolated from patient peripheral blood or bone marrow specimens. DNA is amplified via PCR, the TKD amplicon is enzymatically digested, and the amplicons are detected via capillary electrophoresis on the 3500xL or 3500xL Dx Genetic Analyzer. *FLT3* mutation status is determined by the LeukoStrat CDx *FLT3* Software. A *FLT3* ITD and/or TKD mutation is reported as Positive if the mutant : wild type signal ratio meets or exceeds the cutoff of 0.05 (see section 13: *Interpretation of Results*). A depiction of the workflow is shown in Figure 1.

6.   Principles of the Procedure
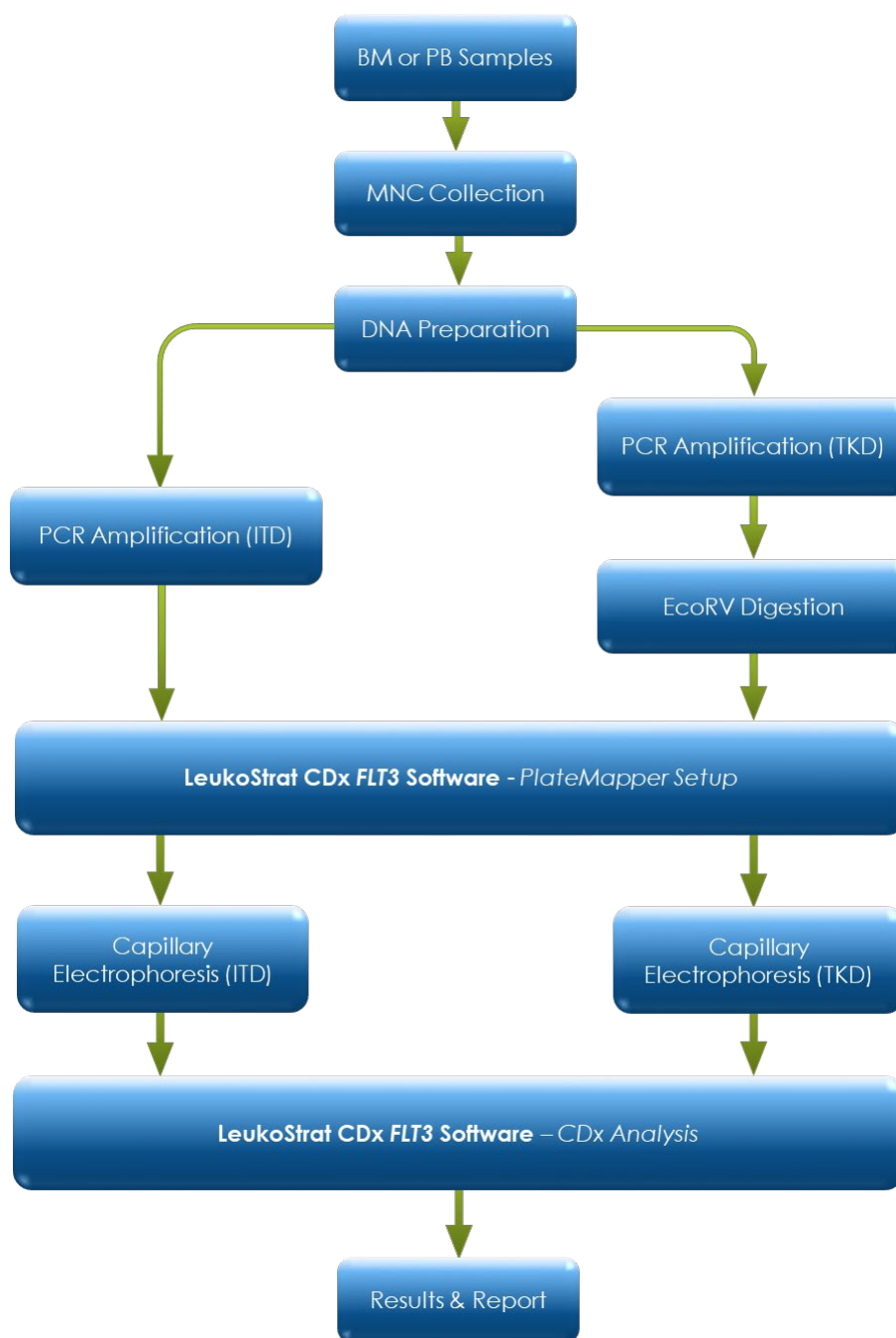
Figure 1: Workflow summary

6.1.   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*.[1]

The LeukoStrat CDx *FLT3* Mutation Assay uses primers that are in and around 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 generate a product measured at 327±1 bp as measured by this assay, while alleles that contain ITD mutations will generate a product that exceeds 327±1 bp (Figure 2).

6.2. 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 2).
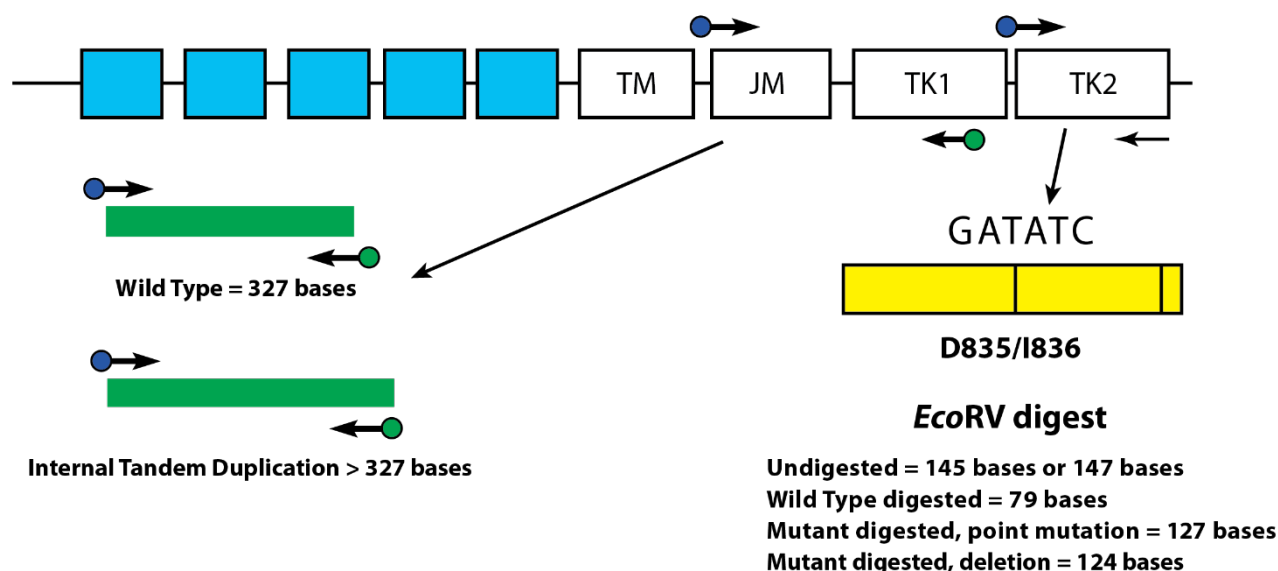
Figure 2: Depicted is a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target in and around the JM region for ITD or the activating loop of the kinase 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.
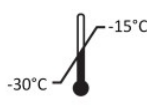
6.3. End-User and In-Use Environment

The Device is for professional use only in a clinical laboratory setting. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the LeukoStrat CDx *FLT3* Mutation Assay.

7. Reagents and Materials

NOTE: The LeukoStrat CDx *FLT3* Mutation Assay kit is usable until the labeled kit expiration date when stored as described in Table 1.

Table 1: Reagent List of the LeukoStrat CDx *FLT3* Mutation Assay Kit, REF K4120431

Catalog Number	Reagent Name	Reagent Label	Storage Temperature	Unit Quantity	# of Units / Kit
REF R0880280 **	*FLT3* Extraction Control	*FLT3* CONTROL EC		1800 µL/Vial	1 Vial
REF B4120151 *	*FLT3* ITD Master Mix	*FLT3* ITD MM		1500 µL/Vial	1 Vial
REF B4120161 *	*FLT3* TKD Master Mix	*FLT3* TKD MM		1500 µL/Vial	1 Vial
REF R0880260 **	*FLT3* ITD Positive Control	*FLT3* ITD CONTROL +	-15°C / -30°C	100 µL/Vial	1 Vial
REF R0880270 **	*FLT3* TKD Positive Control	*FLT3* TKD CONTROL +		100 µL/Vial	1 Vial
REF R0930080 **	*FLT3* No Template Control	*FLT3* CONTROL NTC		200 µL/Vial	1 Vial
REF 261942	Taq DNA Polymerase (Taq)	TAQ		200 µL/Vial	1 Vial
REF 261944	EcoRV Enzyme	EcoRV		200 µL/Vial	1 Vial
REF 261987*	NEBuffer r3.1	NEBr3.1		1250 µL/Vial	1 Vial

*Opened vials of Master Mixes and NEBuffer r3.1 stored frozen may incur up to 4 freeze thaw cycles.
**Opened vials of Controls stored frozen may incur up to 8 freeze thaw cycles.

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

Capillary Platform	Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
3500xL Genetic Analyzer Series	Hi-Di Formamide	ThermoFisher Scientific • Hi-Di™ Formamide (5 mL)	4401457 (1x5mL) 4440753 (4x5mL)	N/A
	LIZ Size Standard	ThermoFisher Scientific: • GeneScan™ 600 LIZ™ dye Size Standard v2.0	4408399	N/A
	Polymer	ThermoFisher Scientific: • POP-7™ Polymer for 3500/3500xL Genetic Analyzers	4393708	N/A
	Buffer	Thermo Fisher Scientific: • Anode Buffer Container (ABC) 3500 Series	4393927	N/A
		Thermo Fisher Scientific: • Cathode Buffer Container (CBC) 3500 Series	4408256	N/A
	Capillary Electrophoresis Instrument and Software	Thermo Fisher Scientific: • 3500xL Genetic Analyzer with Data Collection Software (DCS) v3.3	4405633	This instrument is not CE marked.
		Thermo Fisher Scientific: • GeneMapper™ Software v6.0 full installation with patch "B02" • Official name of "B02" patch: GM_PeakHeight_202110 patch for GeneMapper™Software Full v6.0	A38888	This software is not CE marked. Cat. # is for Windows 10. Request patch from Thermo Fisher Scientific.

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

Capillary Platform	Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
3500xL Genetic Analyzer Series	**Capillary Array**	Thermo Fisher Scientific: • 3500xL Genetic Analyzer 24-Capillary Array, 50 cm	4404689	N/A
	Septa	Thermo Fisher Scientific: • Septa Cathode Buffer Container (for the 3500 series Genetic analyzers)	4410715	N/A
		Thermo Fisher Scientific: • Septa for 3500/3500xL Genetic Analyzers, 96 well	4412614	N/A
	Retainer & Base Set	Thermo Fisher Scientific: Retainer & Base Set (Standard) for 3500/3500xL Genetic Analyzers, 96 well	4410228	N/A
	Spectral Calibration Dye Set	Thermo Fisher Scientific: • DS-33 Matrix Standard Kit (Dye Set G5)	4345833	N/A
3500xL Dx Genetic Analyzer Series	**Hi-Di Formamide**	Thermo Fisher Scientific: • Hi-Di™ Formamide for 3500 Dx/3500xL Dx Genetic Analyzers (CE-IVD)	4440752 (4x5mL) 4404307 (1x5mL)	N/A
	LIZ Size Standard	Thermo Fisher Scientific: • GeneScan™ 600 LIZ™ Size Standard v2.0 Dx	A25794	N/A
	Polymer	Thermo Fisher Scientific: • POP-7™ Polymer for 3500 Dx/3500xL Dx Genetic Analyzers	4393709	N/A
	Buffer	Thermo Fisher Scientific: • Anode Buffer Container for 3500 Dx/3500xL Dx Genetic Analyzers	4393925	N/A
		Thermo Fisher Scientific: • Cathode Buffer Container for 3500 Dx/3500xL Dx Genetic Analyzers	4408258	N/A
	Capillary Electrophoresis Instrument and Software	Thermo Fisher Scientific: • Applied Biosystems 3500xL Dx Genetic Analyzer (24-capillary) with DCS 3.0	A27856	N/A
		Thermo Fisher Scientific: • GeneMapper ™ Software v6.0 full installation with patch "B02" • Official name of "B02" patch: GM_PeakHeight_202110 patch for GeneMapper™Software Full v6.0	A38888	This software is not CE marked. Cat. # is for Windows 10. Request patch from Thermo Fisher Scientific.
	Capillary Array	Thermo Fisher Scientific: • 3500xL Dx Genetic Analyzer 24-Capillary Array, 50 cm	4404688	N/A
	Septa	Thermo Fisher Scientific: • Septa Cathode Buffer Container for 3500 Dx/3500xL Dx Genetic Analyzers	4410716	N/A
		Thermo Fisher Scientific: • Septa for 3500 Dx/3500xL Dx Genetic Analyzers, 96-well	4410700	N/A

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

Capillary Platform	Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
	Retainer & Base Set for 3500xL Dx	Thermo Fisher Scientific: • Retainer & Base Set (Standard) for 3500 Dx/3500xL Dx Genetic Analyzers, 96 well	4410227	N/A
	Spectral Calibration Dye Set	Thermo Fisher Scientific: • DS-33 Matrix Standard Kit Dx (Dye Set G5)	A25775	N/A
Required for Both Capillary Platforms	**Calibrated Pipettes**	• Single Channel 5 – 120 µL • 8 Channel 0.2-10 µL, or equivalent • P-2M, P-10N, P-20N, P100N, P-200N, and P-1000N pipettes, or equivalent	N/A	Must be able to accurately measure volumes between 0.5 µL and 1000 µL.
	Thermal Cycler	Thermo Fisher Scientific: • Veriti™ Dx 96-Well Thermal Cycler, 0.2 mL	4452300	N/A
	Vortex Mixer	N/A	N/A	N/A
	PCR Plates or Tubes	N/A	N/A	Molecular-bio grade Skirted Plates
	Filter Barrier Pipette Tips	N/A	N/A	Molecular bio grade RNase/DNase/Pyrogen-free
	Microcentrifuge	N/A	N/A	N/A
	96-well Aluminum Foil Sheet	N/A	N/A	N/A
	96-well 8-cap Strips	N/A	N/A	N/A
	Glass Distilled De-ionized Molecular Biology Grade or USP Water	N/A	N/A	Sterile, RNase/DNase -free
	DNA Extraction	Qiagen: • QIAamp® DSP DNA Blood Mini Kit	61104	Includes AL buffer, AW1 buffer, AW2 buffer, AE buffer, protease solvent, protease, elution tubes, lysis tubes, and spin columns
	Mononuclear Cell Isolation	Density Gradient Medium	N/A	Density: 1.077 g/mL
	Buffered Saline Solution	Dulbecco's Phosphate-Buffered Saline (DPBS)	N/A	N/A
	Growth Medium	RPMI 1640 with L-glutamine	N/A	N/A
	Cell Counter	N/A	N/A	Capable of counting nucleated cells
	Microvolume UV Spectrophotometer	UV Spectrophotometer	N/A	Capable of measuring absorbance at 260 nm for nucleic acid concentration calculation
	Ethyl Alcohol/Ethanol	N/A	N/A	96-100% Ethanol

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

Capillary Platform	Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
	DNA Extractor System	QIAgen: • QIAcube System (220-240 V) • QIAcube Connect (220 – 240 V)	9001293 9002864	N/A

Table 3: General Laboratory Materials (Not Provided)

Material Description
15 mL conical tubes
50 mL conical tubes
Serological pipettes – 5 mL, 10 mL, 25 mL
Lint free wipes
Calibrated timer
Ice and ice bucket
Container for liquid waste
Volume appropriate non-binding surface tubes for DNA dilutions and aliquots
Volume appropriate tubes for DPBS, PCR, and Digestion Master Mix solutions
Screw caps for QIAcube sample tubes
Disposable transfer pipettes
Pipette tips
96-Well Capillary Electrophoresis Plates, Skirted

8. Instruments/Accessories

NOTE: This assay is for use on the 3500xL or 3500xL Dx Genetic Analyzer and the associated Data Collection Software installed on each instrument.

NOTE: Properly maintain all equipment according to the manufacturer's instructions:

- Refrigerator capable of 2°C to 8°C storage
- Freezer capable of -30°C to -15°C storage
- Dead air box
- 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
- The above instrumentation and accessories are not provided

8.1. Software (Provided)

8.1.1. **REF** : K4120441 includes:

- LeukoStrat CDx *FLT3* Software v1.1.x.IVD
- "3500xL Dx Files" folder containing:

  o *ITD CDx Assay.xml*
  o *TKD CDx Assay.xml*

- "3500xL RUO Files" folder containing:

  o *ITD CDx Assay.xml*
  o *TKD CDx Assay.xml*

Validation of the LeukoStrat software application was performed on display set to 1920 x 1200 resolution, with the "Smaller – 100%" display setting. Display issues may occur at other resolutions.

8.1.1.1. Computer Requirements:
- Operating System: Windows™ 10 Pro or Windows™ 11 Pro
- Processor: Intel Core 2 Duo or newer CPU recommended
- RAM: 4 GB minimum
- Available Disk Space: 5 GB minimum
- A CD-ROM drive
- Adobe Acrobat Reader 2022 or 2023

9. Warnings and Precautions

- Please read the Instructions for Use carefully prior to starting the assay procedure and follow each step closely.
- The assay is only validated for use on the 3500xL or 3500xL Dx Genetic Analyzers the associated Data Collection Software installed on each instrument.
- The assay must be used as a system. Do not substitute other manufacturers' 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.
- Do not mix or combine reagents from kits with different lot numbers.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- Track the number of reagent freeze thaw cycles.
- Perform all 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. Do not pipette by mouth. Do not eat, drink, or smoke in laboratory work areas. Wash hands thoroughly after handling specimens and assay reagents. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets.
- 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. Use fresh, aerosol-resistant pipette tips between samples and

between dispensing reagents. 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 performing PCR.
- Autoclaving does not eliminate DNA contamination. Follow uni-directional work flow in the PCR laboratory between separate work areas; begin with specimen preparation, then to amplification, and finally to detection. Do not bring amplified DNA into the areas designated for 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.
- All instruments and equipment must be maintained and calibrated per the manufacturers' recommendations.
- Once the pouch has been brought to room temperature, examine inside the neck of each POP-7 Polymer pouch at point of installation. Ensure the pouch attachment is free of dried or crystallized polymer. Do not install the pouch on the 3500xL or 3500xL Dx instrument if crystallization is observed, as crystallization may impact the performance of the LeukoStrat CDx *FLT3* Mutation Assay and 3500xL or 3500xL Dx instrument. If crystallization is observed, do not install the pouch on the 3500xL or 3500xL Dx instrument and contact Thermo Fisher customer support.
- Failure of the device to perform as expected or failure to correctly interpret test results may lead to incorrect *FLT3* mutation results and subsequently, improper patient management decisions in AML treatment[3].

  o A false negative assay result could cause an AML patient not to experience any potential benefit that might be associated with receiving gilteritinib fumarate (XOSPATA®) or quizartinib hydrochloride (VANFLYTA®) treatment. However, the patient would receive intense chemotherapy as the standard therapy for AML.
  o Patients with a false positive assay result may receive treatment with gilteritinib fumarate (XOSPATA®) or quizartinib hydrochloride (VANFLYTA®) for which there is no expectation of benefit. For adverse events related to these treatments, refer to the relevant pharmaceutical manufacturer labeling.

NOTE: If incorrect specimens, or reagents are used and/or these instructions are not followed properly, there is a risk of delayed results, which may lead to delay in treatment.

- Any serious incident that has occurred in relation to the device shall be notified to the manufacturer and the Competent Authority in the Member State of the user and/or patient.

9.1. Cyber Security Precautions

The LeukoStrat CDx *FLT3* Software does not require a network connection to function. To minimize cybersecurity risks it is recommended to use the software on a stand-alone computer that is not connected to a network. The following precautions are recommended if a network connected computer is used to host the software.

- Computers and networks are susceptible to security risk if not secured and actively updated. Proper computer and network security helps ensure data is not compromised, lost, or damaged due to preventable cyber risks. Equip all computers with up to date and active antivirus software.
- Filter and secure network traffic with a firewall.
- Keep data on local computers to reduce cyber security risks that may be present in transferring sensitive data over a network.
- Install software only for local user accounts without administrator privileges to prevent unauthorized use of the software.
- Ensure Windows and Adobe Acrobat Reader are always updated to the latest available security patches.
- Remove all non-essential software from the computer and disable web-browser access.
- Ensure that the operating system on the computer locks itself after a period of user inactivity (e.g. 5 mins).
- Only install updates that have been obtained directly from the manufacturer (i.e. Invivoscribe Inc.). Installing security updates follows the same process as software installation.
- It is recommended to back-up the software installation and any results produced by the software to prevent data loss.
- Ensure the default PDF reader in Windows is set to Adobe Acrobat Reader. Opening sample and run reports in an internet browser may lead to cybersecurity risks of patient data.
- The LeukoStrat CDx *FLT3* Software has been validated with the following Antivirus software:

  o Symantec Endpoint Protection Version 14.3
  o McAfee Endpoint Security Version 10.7
  o ESET Endpoint Security Version 10.0

10. Specimen Collection and Preparation

10.1. Precautions

- Biological specimens from humans may contain potentially infectious materials. Handle all specimens according to your institute's Bloodborne Pathogen program and/or Biosafety Level 2.
- The assay is validated for blood and bone marrow anti-coagulated with sodium heparin or EDTA.

10.2. PCR-Interfering Substances

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)

10.3. Specimen Requirements and Handling

- At least 1 mL of peripheral blood or 0.25 mL of bone marrow anti-coagulated with sodium heparin or EDTA are required for the LeukoStrat CDx *FLT3* Mutation Assay.
- Specimens can be stored at 2°C to 8°C for up to 7 days prior to testing.
- Specimen tube integrity and contents must not be compromised (*i.e.* frozen during shipment).

11. Assay Procedure

11.1. Specimen Inspection

11.1.1. Unpack peripheral blood (PB) and/or bone marrow aspirate (BM) specimens and reject any specimens that do not meet the requirements listed in section 10.3.

11.2. Sample Processing Preparation

11.2.1. Perform sample processing in an assigned sample processing workspace.

11.2.2. Transfer approximately 14 mL of RPMI-1640 media per specimen into labeled 50 mL conical tubes. Allow the media to warm at room temperature for at least 1.75 hours.

- If chilled RPMI-1640 media is aliquoted into 15 mL conical tubes, warm at room temperature for at least 45 minutes.

11.2.3. For each specimen, aliquot 3 mL of density gradient medium into a labeled 15 mL conical tube.

- If the density gradient medium was stored at 2°C to 8°C, warm the density gradient medium aliquots at room temperature for 1 hour before use.

11.2.4. Transfer approximately 200 μL of DPBS per specimen into a labeled, volume appropriate tube and allow it to warm at room temperature for at least 45 minutes prior to use.

11.3. Diluting Clinical Samples

NOTE: Instructions for using a QIAcube for DNA extraction are included in this manual. A QIAcube is recommended but not required. If a QIAcube is used, ensure that one space is reserved for the Extraction Control.

11.3.1. Mix the specimen tubes by inverting 4 to 6 times. Add specimen aliquots (1-3 mL peripheral blood or 0.25-0.75 mL bone marrow) to uniquely labeled 15 mL conical tubes.

11.3.2. Add RPMI-1640 media to each specimen aliquot to bring the total volume to 6 mL. Cap the tubes tightly and gently mix by inverting 3 to 5 times or pipette up and down until the mixture has a uniform consistency.

11.3.3. Any remaining specimen may be stored at 2°C to 8°C.

11.4. Mononuclear Cell (MNC) Isolation

11.4.1. Using a transfer pipette, gently overlay the diluted peripheral blood or bone marrow sample on top of the density gradient medium. Tilt the tube containing the density gradient medium while very slowly pipetting the sample on top to prevent layer mixing.

11.4.2. After pipetting the entire sample, gently straighten the tube to a vertical position and cap the tube tightly.

11.4.3. Centrifuge the 15 mL conical tubes under the following conditions, ensuring that the brake is completely turned off:

- Force = 400 x g (rcf)
- Time = 30 minutes

- ▪ Temperature = 20°C
- ▪ Accel/Decel = minimum

11.4.4. For each sample to be processed, aliquot 6 mL of RPMI-1640 media into a new labeled 15 mL conical tube.

11.4.5. After centrifugation, use a transfer pipette to slowly aspirate the MNC layer or until no more than 3 mL has been removed.

11.4.6. Dispense the collected MNC layer suspension into the appropriately labeled 15 mL conical tube containing 6 mL of RPMI-1640 media. Cap the tube and gently mix by inverting 3 to 5 times.

11.4.7. Centrifuge the conical tubes under the following conditions:

- ▪ Force = 355-364 x g (rcf)
- ▪ Time = 10 minutes
- ▪ Temperature = 20°C
- ▪ Accel/Decel = maximum

11.4.8. Pour the supernatant off the cell pellet by only inverting the tube once before returning it to the vertical position. Resuspend the pellet in the remaining liquid by tapping the tube 10 to 15 times or until the pellet is resuspended.

11.4.9. Add 1 mL of RPMI-1640 media to the resuspended cell pellet. Cap the tube and gently mix by tapping the tube 6 to 8 times.

11.4.10. Place the sample tubes in an ice water bath until the mononuclear cell counts have been completed.

11.5. Mononuclear Cell Count

11.5.1. Obtain mononuclear cell counts using a suitable cell counting system.

11.5.2. Minimize the volume consumed for cell counts to ensure an adequate amount of DNA is retained for the assay.

- ▪ Discard the sample used for cell counting.

11.6. Preparing Samples for DNA Extraction and Isolation Completion

11.6.1. If the reported concentration is ≤ 5 million cells/mL, the entire cell suspension volume is processed. Proceed to step 11.6.3.

11.6.2. If the reported concentration is > 5 million cells/mL, calculate the volume of sample that contains 5 million live cells (V_i) since the QIAcube spin columns can only accommodate ≤ 5 million cells.

11.6.2.1. Use the equation $C_iV_i = C_fV_f$, to solve for V_i for each of these samples.

- ▪ C_i = cell concentration (cells/mL) from the MNC count
- ▪ C_f = final concentration (5 million cells/mL)
- • V_f = final volume (1 mL)
- • $V_i = \dfrac{5{,}000{,}000\frac{cells}{mL} x\ 1\ mL}{C_i}$

11.6.2.2. Use the equation $V_f - V_i$ to solve for the volume of RPMI-1640 media to add to V_i to bring the volume up to 1000 µL.

11.6.2.3. Gently mix the tubes containing the > 5 million cells/mL samples by tapping the tubes 6 to 8 times.

11.6.2.4. Transfer the calculated volumes to a labeled 15 mL conical tube for each sample.

11.6.3. Centrifuge the 15 mL conical sample tubes containing the cell suspensions under the following conditions:

- ▪ Force = 355-364 x g (rcf)
- ▪ Time = 10 minutes
- ▪ Temperature = 20°C
- ▪ Accel/Decel = maximum

11.6.4. Using a transfer pipette, aspirate the supernatant from the cell pellets. Some small volume of media may remain.

11.6.5. Tap the 15 mL conical tubes 10 to 15 times or until the pellets are loosened from the tubes.

11.6.6. Add 200 µL of DPBS and gently mix by tapping the tube 10 to 15 times to resuspend the cells. Place these capped samples in the ice water bath.

11.7. Preparing QIAcube Automation Station

NOTE: Instructions for use of a QIAcube for DNA extraction are included in this manual. A QIAcube is recommended but not required. DNA extraction can be performed with the Qiagen DSP DNA Blood Mini Kit without a QIAcube.

11.7.1. All QIAcube Automation Station steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.

11.7.1.1. Follow the QIAgen guidelines for performing maintenance on the QIAcube Automation Station, with one exception. Perform the Tightness Test monthly instead of every 6 months.

11.7.2. Prepare the QIAcube Automation Station for use, loading materials and reagents into the instrument.

11.7.2.1. A QIAcube is capable of processing up to 12 tubes; however, one of the spaces is reserved for the Extraction Control (used as the extraction contamination control and PCR negative control). It is not possible to process 1 or 11 tubes due to centrifuge imbalance.

11.7.2.2. Blank tubes, using DPBS, may be used if the number of required extractions including Extraction Control is 11 tubes.

11.7.3. Remove a tube of Extraction Control (EC) from -30°C to -15°C storage and thaw at room temperature. Control EC tubes may be returned to the freezer after use. Track the number of freeze thaw cycles.

11.7.4. Vortex the EC tube at MAX speed for 5 to 15 seconds. Centrifuge the tube for 2 to 5 seconds if liquid is present in the lid. Add 200 µL of the Extraction Control to a sample tube. This EC tube may be capped and stored at 2°C to 8°C until the run is ready.

11.8. DNA Extraction

NOTE: Instructions for using a QIAcube for DNA extraction are included in this manual. A QIAcube is recommended but not required. DNA extraction can be performed with the Qiagen DSP DNA Blood Mini Kit without a QIAcube.

11.8.1. Pipette the cell suspensions (from step 11.6.6) up and down 4 to 6 times to resuspend the cells. Transfer the entire volume of the cell suspensions in DPBS to sample tubes. Ensure that the majority of the solution is at the bottom of the tube.

11.8.2. Place the Extraction Control sample tube in the last position of the run.

11.8.3. Load all of the remaining sample tubes, the reagents, and the aliquoted Protease solution into the instrument.

11.8.4. Start the run, ensuring the following selections are made.

11.8.4.1. Use the QIAamp DNA Blood Mini protocol.

11.8.4.2. Select **Blood** or **body fluid** as the starting material.

11.8.4.3. Set the *Elution volume* to **100 µL**.

11.8.5. When the extraction is complete, cap the DNA sample tubes and store them at 2°C to 8°C until quantification is performed.

11.9. Quantification and Dilution of DNA

11.9.1. All microvolume UV spectrophotometer steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.

11.9.2. Vortex the DNA sample tubes at MAX speed for 5 to 15 seconds. Using a microcentrifuge, centrifuge the DNA sample tubes for 2 to 5 seconds to remove liquid from the lids.

11.9.3. Blank the instrument using 2 µL of AE buffer.

11.9.4. Read 2 µL of each DNA sample in singlicate.

11.9.5. If the concentration of a DNA sample has a reading ≤9.4 ng/µL, re-quantify the DNA sample twice more using fresh 2 µL aliquots. Ensure the sample is well mixed to avoid inaccurate microvolume UV spectrophotometer readings. The average of these three readings is considered the final DNA concentration.

NOTE: If the final quantification value is ≤9.4 ng/µL, the DNA sample cannot be tested in the LeukoStrat CDx *FLT3* Mutation Assay. Reprocess the specimen in order to obtain adequate DNA.

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.

11.9.6. DNA samples may be stored undiluted at -30°C to -15°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 may be exposed to up to five (5) freeze thaw cycles.

11.9.7. DNA samples ≥10.5 ng/µL must be diluted to 10 ng/µL in AE buffer using non-binding surface tubes. Using the equation $C_iV_i = C_fV_f$, solve for V_i after selecting the final volume (V_f) from Table 4.

▪ $$V_i = \frac{\left(V_f \ x \ 10\frac{ng}{\mu L}\right)}{c_i}$$

- C_i = DNA concentration from microvolume UV spectrophotometer reading
- C_f = final DNA concentration (10 ng/µL)
- V_i = volume of undiluted DNA to dilute
- V_f = final volume of diluted DNA (from Table 4)
- V_f - V_i = amount of AE buffer to add to V_i

Table 4: Final Volumes Determination by Quantification Value

DNA Concentration from Microvolume UV Spectrophotometer *(C_i)*	Final Volume *(V_f)*
C_i ≤ 9.4 ng/µL	Not testable
9.5 ≤ C_i ≤ 10.4 ng/µL	Test as is
10.5 ≤ C_i ≤ 50.4 ng/µL	35 µL
50.5 ≤ C_i ≤ 200.4 ng/µL	100 µL
C_i ≥ 200.5 ng/µL	180 µL

11.10. Amplification

NOTE: Complete all steps in this section on the same day for an ITD or TKD run.
NOTE: Minimize the amount of light exposure to the Master Mixes.
NOTE: Minimize the amount of time Taq is out of -30°C to -15°C storage.

11.10.1.   Perform all Veriti Dx thermal cycler steps including installation, operation, calibration, cleaning and maintenance procedures according to the manufacturer's instructions unless stated otherwise below.

11.10.2.   Allow Master Mixes (ITD Master Mix and TKD Master Mix) to thaw at room temperature. Remove Control tubes (ITD Positive Control, TKD Positive Control, Extraction Control, and No Template Control) from appropriate storage and thaw at room temperature. Return control tubes to the freezer after use, tracking the number of freeze thaw cycles. While reagents are warming to room temperature, label separate 96-well plates with ITD PCR or TKD PCR, as applicable, and a unique identifier.

NOTE: Run all samples on the same PCR plate as the associated Extraction Control.

11.10.3.   Determine the number of plate wells (samples, TKD Positive Controls, ITD Positive Controls, Extraction Controls, and No Template Control) to be tested on the ITD and TKD plates. The total number of plate wells to be tested per ITD or TKD plate = X. To prevent variation when pipetting small volumes of reagent, the minimum value of X is two (2).

11.10.3.1.   Calculate the volumes of master mix and Taq needed:

- Total volume of master mix $=$ 45 µL $\times$ (X + 3)
- Total volume of Taq $=$ 0.2 µL $\times$ (X + 3)
- The additional three (3) samples added to X allow for pipetting error.

11.10.4.   Vortex the Master Mix, Controls, and DNA sample tubes at MAX speed for 5 to 15 seconds.

11.10.5.   Remove Taq from storage at -30°C to -15°C. Do not vortex.

11.10.6.   Using a microcentrifuge, centrifuge all tubes (including Taq) for 2 to 5 seconds to remove liquid from the lids.

11.10.7.   Add the calculated volumes of Master Mix and Taq to labeled tubes of the appropriate volume for the ITD and TKD plates.

11.10.8.   Cap and vortex the tubes at MAX 5 to 15 seconds to mix. Use a microcentrifuge to centrifuge for 2 to 5 seconds, when possible. Place Taq back in storage at -30°C to -15°C.

11.10.9.   Aliquot 45 µL of the mixture of Master Mix and Taq to the appropriate wells of the PCR plate layout.

11.10.10. Add 5 µL of the DNA samples at 10 ng/µL and Controls to the appropriate wells of the 96-well plate according to the PCR plate layout.

11.10.11. Seal the columns of the PCR plate with well strips. Centrifuge the 96-well plate at 1400×g for 1 minute.

11.10.12. Place the PCR plate in a Veriti Dx thermal cycler and close the lid. Program the thermal cycler with the steps listed in Table 5.

Table 5: PCR Amplification Thermal Cycler Programs

Step	*FLT3* ITD CDx Program	*FLT3* TKD CDx Program
1	95°C for 11 minutes	94.5°C for 11 minutes
2	94°C for 30 seconds	93.5°C for 30 seconds
3	57°C for 60 seconds	56.5°C for 60 seconds
4	72°C for 2 minutes	71.5°C for 2 minutes
5	Repeat Steps 2 to 4, 24 times	Repeat Steps 2 to 4, 28 times
6	94°C for 30 seconds	93.5°C for 30 seconds
7	60°C for 45 minutes	59.5°C for 45 minutes
8	4°C ∞	4°C ∞
	Ramp rate 75%.	

11.10.13. Press **Run** to proceed to the next screen. Ensure that the reaction volume setting is *50 μL*, the cover temperature setting is *105.0 °C*, and that the cover will be heated for the run. Press **Start Run Now** to begin the run.

11.10.14. Store leftover reagents and DNA. Store opened Master Mixes at -30°C to -15°C. Track the number of freeze thaw cycles.

11.10.15. After completion of the PCR protocol, the PCR plate may be stored at 2°C to 8°C for up to 72 hours. Otherwise, for TKD plates, continue to section 11.11: *Restriction Digest (TKD Mutation Only)* and for ITD plates, continue to section 11.12: *Capillary Electrophoresis Detection.*

11.11. Restriction Digest (TKD Mutation Only)

NOTE: Complete all steps in this section in the same day.
NOTE: Perform the restriction enzyme digest on the TKD amplicons only.
NOTE: Minimize the amount of time EcoRV is out of -30°C to -15°C storage.

11.11.1. Allow a NEBuffer r3.1 tube to thaw at room temperature.

11.11.2. While reagents are warming to room temperature, label a 96-well plate with TKD digestion and a unique identifier.

11.11.3. Determine the number of plate wells (samples and controls) to be digested on the plate. The total number of samples to be digested = Y.

- To prevent variation when pipetting small volumes of reagent, the minimum value of Y is four (4).

11.11.3.1. Calculate the volumes of NEBuffer r3.1 and EcoRV needed.

- Total volume of NEBuffer r3.1 = 1.1 μL × (Y + 6)
- Total volume of EcoRV = 0.5 μL × (Y + 6)
- The additional six (6) samples added to Y allow for pipetting error.

11.11.4. Vortex the NEBuffer r3.1 tube at MAX for 5 to 15 seconds.

11.11.5. Remove the EcoRV from the -30°C to -15°C storage. Do not vortex.

11.11.6. Using a microcentrifuge, centrifuge all tubes (including EcoRV) for 2 to 5 seconds to remove liquid from the lids.

11.11.7. Add the calculated volumes of NEBuffer r3.1 and EcoRV to a labeled tube of appropriate volume.

11.11.8. Mix the solution by pipetting up and down 5 to 10 times. Place EcoRV back in storage at -30°C to -15°C.

11.11.9. Aliquot 1.5 μL of the digestion mix solution to the appropriate wells of the digestion plate.

11.11.10. Remove the TKD PCR plate from the thermal cycler or 2°C to 8°C storage (plate does not need to warm to room temperature) and centrifuge the plate at 1400×g for 1 minute.

11.11.11. Add 8.5 μL of the samples from the PCR plate to the appropriate wells of the digestion plate. Seal the digestion plate columns with cap strips.

11.11.12. Centrifuge the plate at 1400×g for 1 minute.

11.11.13. Place the digestion plate in a Veriti Dx thermal cycler and close the lid.

11.11.14. Program the thermal cycler with the steps listed below (ramp rate 75%).

- Step 1: 37°C for 1 hour
- Step 2: 65°C for 10 minutes
- Step 3: 4°C for ∞

11.11.15.   Press **Run** to proceed to the next screen. Ensure that the reaction volume setting is *10 µL*, the cover temperature setting is *105.0°C*, and that the cover will be heated for the run. Press **Start Run Now** to begin the run.

11.11.16.   After completion of the digestion protocol, the digestion plate may be stored at 2°C to 8°C for up to 72 hours, keeping the light exposure to a minimum. Otherwise, continue to *Capillary Electrophoresis Detection* (section 11.12).

11.12.   Capillary Electrophoresis Detection

NOTE: Minimize the amount of time the LIZ Size Standard tube is out of 2°C to 8°C storage.

NOTE: The 3500xL and 3500xL Dx run in sets of 24 capillaries, called an injection, which comprises three (3) columns by eight (8) rows on a 96-well plate. Each capillary corresponds with one well. There are no partial injections although the injections can be independently programmed.

11.12.1.   All 3500xL and 3500xL Dx steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.

11.12.2.   ITD and TKD assays must be run on different injections and with different injection conditions. The 3500xL and 3500xL Dx ITD and TKD conditions are listed in Table 6 below. These settings are provided as XML files which can be imported into the instruments and saved on the ABI 3500xL and 3500xL Dx for future use.

Table 6:   3500xL and 3500xL Dx Genetic Analyzer Conditions

Parameter	ITD CDx Assay Parameters	TKD CDx Assay Parameters
Injection Time	12 sec.	7 sec.
Injection Voltage	1.2 kVolts	1.0 kVolts
Capillary Length	50 cm	
Polymer	POP-7	
Dye Set	G5	
Oven Temp	60°C	
Run Time	1630 sec.	
Run Voltage	19.5 kVolts	
PreRun Time	180 sec.	
PreRun Voltage	15 kVolts	
Data Delay	1 sec.	

11.12.3.   Click **Refresh** to update the consumables' time on the instrument and number of injections performed in the 3500xL or 3500xL Dx Dashboard. Check the 3500xL or 3500xL Dx dashboard to ensure that the buffers, polymer, and capillary have not exceeded their maximum allowable time on the instrument for this assay, listed in Table 7. Check that the number of samples (not just injections) remaining for POP-7 is sufficient for the run. If a consumable component needs to be replaced, perform the necessary maintenance before proceeding.

Table 7:   Maximum Allowable On-Board Times, 3500xL or 3500xL Dx Materials

3500xL (Dx) Material	Maximum Allowable Time on Instrument
POP-7 Polymer	7 days
Anode Buffer	7 days
Cathode Buffer	7 days
3500xL Capillary Array or 3500xL Dx Capillary Array	400 injections or 160 injections

11.13.   Prepare Size Standard Solution, if needed

11.13.1.   The size standard solution consists of a mixture of LIZ Size Standard and Hi-Di Formamide.

11.13.2.   Remove a tube of size standard solution from 2°C to 8°C storage, if available, and proceed to step 11.13.6. If not, make a size standard solution tube by performing the next three steps.

11.13.3.   Allow a bottle of Hi-Di Formamide to thaw at room temperature. Remove a tube of LIZ Size Standard from storage.

11.13.4.   Vortex the tubes at MAX for 5 to 15 seconds. Centrifuge the tubes for 2 to 5 seconds in a microcentrifuge.

11.13.5.   Add 56 µL of LIZ Size Standard to 1 mL of Hi-Di Formamide. Label the size standard solution tube with the date.

11.13.6. Vortex the size standard solution tube at MAX for 5 to 15 seconds. Centrifuge the mixture tube for 2 to 5 seconds in a microcentrifuge. Any unused solution can be stored at 2°C to 8°C for up to 7 days. Discard after 7 days.

11.14. Prepare Sample Plate

11.14.1. Centrifuge the 96-well ITD PCR and/or TKD digestion plate at 1400×g for 1 minute.

11.14.2. Label a 96-well plate with ITD CE and/or TKD CE, as applicable, and a unique identifier.

NOTE: Both ITD and TKD assays may be run on the same plate, but they must be separated into different injections.

11.14.3. Determine the number of wells needed for a run.

- Number of wells = 24X
    - o X = the number of injections.
- Calculate the volume of size standard solution needed.
    - o Maximum volume of size standard solution = 9.5 µL × (24X + 4)
    - o The additional four (4) samples added to X allow for pipetting error.

11.14.4. Add 9.5 µL of size standard solution to the wells in the CE plate that contain samples. Add 9.5 µL of size standard solution or Hi-Di Formamide only to any remaining wells that will be injected (multiple of 24) but do not contain samples.

NOTE: All 24 wells within an injection must contain sample mixed with size standard solution, size standard solution alone, or Hi-Di Formamide alone.

11.14.5. From each PCR well (ITD only) or digestion well (TKD only), transfer 0.5 µL of PCR or digested product to the corresponding well on the CE plate using a multichannel pipette.

NOTE: A single channel pipette may be used to transfer PCR/digested product during retests of individual wells.

11.14.6. Seal the CE plate with a foil seal and centrifuge the plate at 1400×g for 1 minute.

11.14.7. Place the CE plate in a Veriti Dx thermal cycler and close the lid.

11.14.8. Program the thermal cycler with the steps listed below (ramp rate 75%).

- Step 1: 95°C for 3 minutes
- Step 2: 4°C for 5 minutes

11.14.9. Press **Run** to proceed to the next screen. Ensure that the reaction volume setting is *10 µL*, the cover temperature setting is *105.0°C*, and that the cover will be heated for the run. Press **Start Run Now** to begin the run.

11.14.10. After the run is complete, confirm no bubbles are present by visually inspecting the plate wells. Remove any bubbles by centrifuging the CE plate at 1400×g for 1 minute.

11.14.11. Prepare each plate assembly by placing the CE plate on a 3500xL or 3500xL Dx 96-well plate base, confirming the notched corners align. Remove the foil seal and place a new 96-well plate septa on the plate, ensuring the septa is flat and all septa openings are unobstructed. Snap on a 3500xL or 3500xL Dx 96-well plate retainer.
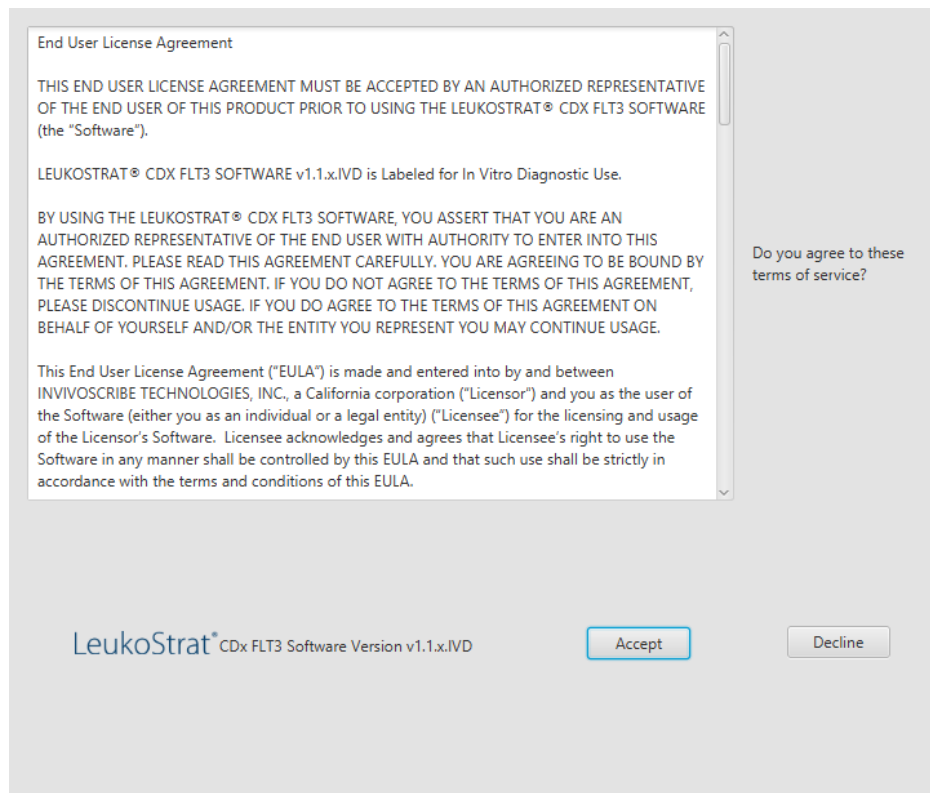
11.15. Set up PlateMapper with LeukoStrat CDx *FLT3* Software

NOTE: Administrator permission is required to install the LeukoStrat® CDx *FLT3* Software.

11.15.1.   Install the LeukoStrat CDx *FLT3* Software.

11.15.1.1.   Copy *LeukoStratCDx-1.1.x.IVD.msi* installer from the software CD to a local drive on your computer.

11.15.1.2.   Double-click the **LeukoStratCDx-1.1.x.IVD.msi** file.

11.15.1.2.1. If a *Microsoft Defender SmartScreen* message appears after double-clicking the msi file, Click **More info**.

11.15.1.2.2. Verify the publisher is Invivoscribe, Inc. To proceed with installation, click **Run anyway**.

11.15.1.3.   The *LeukoStratCDx-1.1.x.IVD.msi* setup wizard box will appear. Click **Next**.

11.15.1.4.   The default installation location is *C:\Invivoscribe\LeukoStratCDx-1.1.x.IVD*. Click **Next**.

11.15.1.5.   Click **Install**. The installation will begin.

11.15.1.6.   A *User Account Control* dialog box will appear. Click **Yes**.

11.15.1.7.   Click **Finish** to exit the setup wizard.

11.15.2.   Open the *LeukoStrat CDx FLT3 Software*. Click **Accept** (Accept) to agree to terms of service.

End User License Agreement

THIS END USER LICENSE AGREEMENT MUST BE ACCEPTED BY AN AUTHORIZED REPRESENTATIVE OF THE END USER OF THIS PRODUCT PRIOR TO USING THE LEUKOSTRAT® CDX FLT3 SOFTWARE (the "Software").

LEUKOSTRAT® CDX FLT3 SOFTWARE v1.1.x.IVD is Labeled for In Vitro Diagnostic Use.

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Do you agree to these terms of service?

LeukoStrat® CDx FLT3 Software Version v1.1.x.IVD   Accept   Decline

11.15.3. Within the *PlateMapper Setup*, fill in the three required fields located above the plate map. These required fields are *Plate Name*, *Results Group*, and *File Name Convention* (circled below).
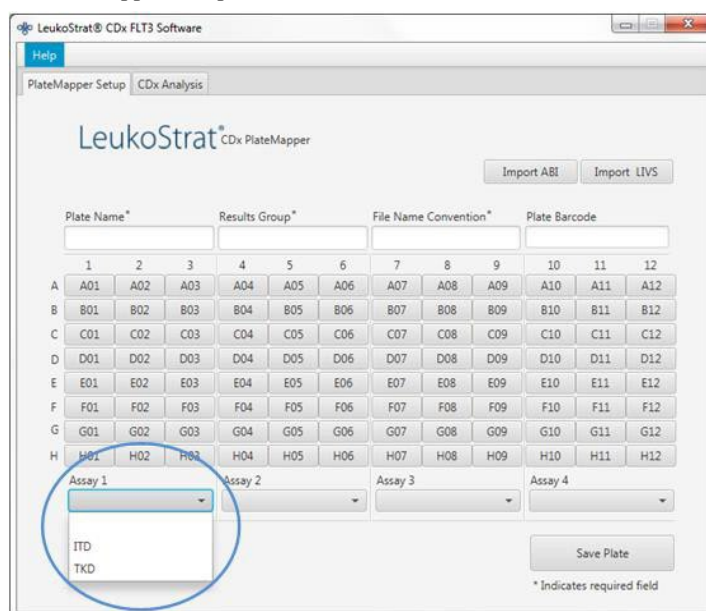
    11.15.3.1. Plate map names may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.

    11.15.3.2. *Results Group* and *File Name Convention* entries must match the names of corresponding user-programmed entries on the 3500xL or 3500xL Dx (selected in step 11.16.14).

11.15.4. The plate map has four assays allowed per plate (three columns per assay). Each assay corresponds with the injection that will occur during the 3500xL or 3500xL Dx run. Only one assay may be run per injection (ITD or TKD).

11.15.5.   Select the assay from the drop down menu () which corresponds with the samples that are located above it on the *PlateMapper Setup* screen.

11.15.6.   Within the plate map, enter information for each well that will have a sample or control to be analyzed.

NOTE: When entering well information, enter the *Extraction Control (EC), Positive Control (PC),* and *No Template Control (NTC)* first. Controls may be placed anywhere on the plate, not necessarily in the first three wells. Enter the information for the SAMPLE wells afterwards, as they will require linking with their corresponding Extraction Control. Positive Controls and No Template Controls are not linked to Extraction Controls.

11.15.6.1.   To enter information, click on the respective well (*e.g.* A01) and the following box will open:

11.15.7.   Enter a *Sample Name* that describes the well. Sample names may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.

11.15.7.1.   The user may also import sample names to the plate map using the *3500 Plate Layout File Version 1.0* by Thermo Fisher Scientific. Enter sample names into the *3500 Plate Layout File* and import with the **Import ABI** button.

11.15.8.   Select the **Sample Type** for each well from the drop down menu. The options to select are:

- SAMPLE = unknown
- EC = Extraction Control
- NTC = No Template Control
- PC= Positive Control

11.15.9.   Select the **Run Number** from the drop down menu. To add a new run number, click the "+" sign next to the drop down menu.

NOTE: A "run" is defined by all samples, one replicate of Positive Control, all Extraction Controls associated with the samples under test, and one replicate of No Template Control. Runs may span multiple injections and multiple runs may be tested on one plate.

11.15.9.1.   Select the associated **EC** from the drop down menu (only required if the *Sample Type* is *SAMPLE*). Up to 11 samples may be associated with a single Extraction Control.

11.15.10.   Additional comments about the sample or control may be entered in the *Sample Notes* field. These comments will appear in the *Sample Report*.

11.15.11. Once all the information for the well has been entered, click **Save Well** to save. To clear the contents of the well, click **Clear Well**.

11.15.12. Once the well has been saved, the color displayed will change for that well. The well will display as red until it has been set up correctly, at which point it will change to green (as seen below).

NOTE: If correct, the well color for the Extraction Control will not change to green until the cursor hovers over that well.

11.15.13. Continue entering each well on the *PlateMapper Setup* screen until all wells with sample information are highlighted green.

11.15.14. Once all wells have been entered, click **Save Plate,** and the user will be prompted for a location to save the ABI file (3500 Plate Layout File Version 1.0) and LIVS file generated by the software. One ABI file and one LIVS file will be generated for every plate setup.

NOTE: Do not modify the ABI file generated by the LeukoStrat CDx *FLT3* Software. Doing so will result in an error upon upload to the 3500xL or 3500xL Dx.

NOTE: If the LeukoStrat CDx *FLT3* Software is not closed once a plate map is generated, the automatically assigned Run IDs in the output files will not be unique and will repeat across multiple runs.

11.15.14.1. The user may review the LIVS file created by clicking on **Import LIVS** and navigating to the file location where it was saved.

NOTE: The **Import LIVS** feature is only to review plate setup. The LIVS file cannot be modified to create a new plate map for use in another run. Doing so will result in an error.
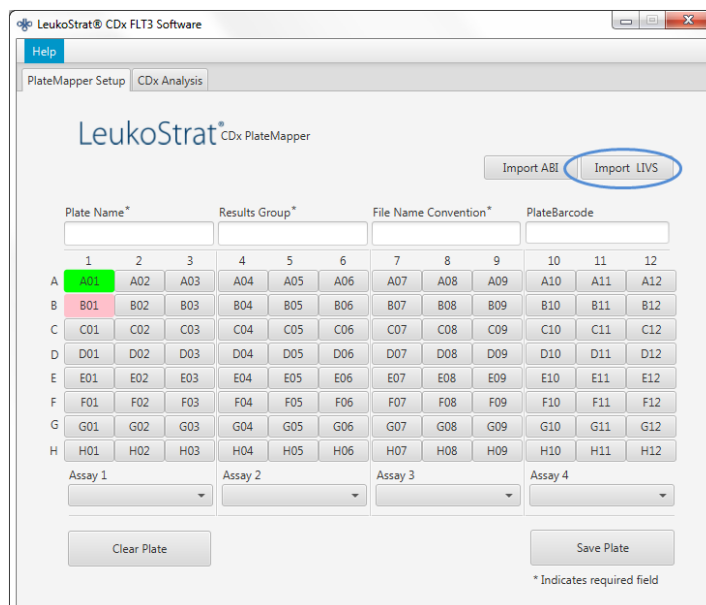
11.15.15. The user will continue with the software after the 3500xL or 3500xL Dx run has completed.

11.15.16. Use the ABI file generated by the LeukoStrat CDx *FLT3* Software to upload the plate onto the 3500xL or 3500xL Dx.

11.15.17. If saving the plate fails, follow the recommendations in Table 8. If further assistance is required, please contact Invivoscribe Technical Support at support@invivoscribe.com.

Table 8: Save Plate Error Messages and Corrective Actions

Save Plate Error Message [code]	Potential Cause(s)	Corrective Action(s)
• Corrupted sample detected. [PM01] • Could not detect well for object UUID. [PM02] • Control detected unknown links for well (A-H, 01- 12). [PM3]	Attempt to upload modified LIVS file.	Do not modify the LIVS file. If the file is corrupted, a new LIVS file must be created.
• Missing required field "Plate Name". [PM04] • Illegal character detected in "Plate Name". [PM05] • Multiple spaces detected in "Plate Name". [PM06] • Plate Name must be 50 characters or less. [PM28]	Not following IFU directions for naming a plate.	A plate map name may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.
• Missing required field "Result Group". [PM07]	Not following IFU directions for naming a *Result Group*.	The *Result Group* is defined on the 3500xL or 3500xL Dx.
• Missing required field "File Naming Convention". [PM08]	Not following IFU directions for naming a *File Naming Convention.*	The *File Naming Convention* is defined on the 3500xL or 3500xL Dx.
• Assay not selected for all samples. [PM09] • Run contains more than 1 Assay type. [PM10]	Not following IFU directions for assigning assay type.	All wells must be assigned an assay type, and a run may not contain more than one assay type.
• Sample name not detected for well (A-H, 01-12). [PM11] • Illegal character detected in Sample Name. [PM12] • Multiple spaces detected in Sample Name. [PM13] • Sample name must be 50 characters or less. [PM14]	Not following IFU directions for naming a sample.	A sample name may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.

Table 8: Save Plate Error Messages and Corrective Actions

Save Plate Error Message [code]	Potential Cause(s)	Corrective Action(s)
• Sample Type not selected for well (A-H, 01-12). [PM15]	Not following IFU directions for selecting a sample type.	All wells must have a sample type assigned. The choices are *PC*, *NTC*, *EC*, and *SAMPLE*.
• Run not selected for well (A-H, 01-12). [PM16] • No Runs created for Plate. [PM17]	Not following IFU directions for selecting runs.	All wells must have a run assigned. The first well assigned a run requires the user to increment the run count ("+" button next to *Run* selection). Subsequent wells can either increment the run count, or select a previously used run count.
• EC not selected for well (A-H, 01-12). [PM18] • Sample attached to unknown EC for well (A-H, 01- 12). [PM19] • EC selected on control for well (A-H, 01-12). [PM20] • No samples linked to EC for well (A-H, 01-12). [PM21]	Not following IFU directions for assigning ECs. Attempt to upload a modified LIVS file.	All *SAMPLE* wells must be assigned to an EC. Do not assign a control well to an EC. Each EC must be linked to at least one sample.
• Run missing PC, NTC, EC. [PM22] • Run detected control in sample list. [PM23] • Run missing samples. [PM24] • Run contains more than one (1) assay type. [PM25]	Not following IFU directions for assigning runs. Attempt to upload a modified LIVS file.	Each run must contain one of each control type (PC, NTC, EC). A run must contain at least one well with type *SAMPLE*. A run must contain exactly one Assay type.
• Too many samples linked to EC for well (A-H, 01- 12). [PM26] • EC linked to more than one run for well (A-H, 01- 12). [PM27]	Not following IFU directions for assigning ECs.	A single EC may not be linked to more than 11 samples. A single EC may not be linked to samples across more than one run.

11.16. Set Up 3500xL or 3500xL Dx Software

NOTE: The LeukoStrat CDx *FLT3* Software creates a file for import into the 3500xL or 3500xL Dx (ABI file) which appends information to the Sample Name. The 3500xL or 3500xL Dx software may append additional information.

11.16.1. All 3500xL and 3500xL Dx steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.

11.16.2. If the Data Collection Software does not already contain ITD CDx Assay and TKD CDx Assay settings, import the files provided on the accompanying Software CD into the ABI 3500xL or the IVD mode of the ABI3500xL Dx software according to the instrument's User Guide.

NOTE: On the provided software disc, there are two folders containing .xml files: 3500xL RUO Files and 3500xL Dx Files; select the appropriate files for import based on the instrument which is being used. Attempting to import the wrong files into either instrument may result in an error.

NOTE: Having an Assay, File Name Convention, or Results Group with the same name in the RUO mode and IVD mode of the 3500xL Dx Data Collection Software may result in an error. Ensure that names matching the provided XML file names do not exist in the RUO mode of the 3500xL Dx DCS.

11.16.3. From the 3500xL or 3500xL Dx dashboard screen, click on the **Create New Plate** button.

11.16.4. For the *Plate Name*, enter a short descriptor.

11.16.5. Ensure the number of wells is set to *96*.

11.16.6. For the plate type, select **Fragment** from the drop down menu.

11.16.7. Ensure the capillary length is *50 cm*, and the polymer is *POP7*.

11.16.8. Enter the operator initials in the *Owner* section.

11.16.9. Click Assign Plate Contents.

11.16.10. Click the **Import** button at the top of the screen and a popup window appears. Navigate to the *3500xL (Dx) import file* (the ABI file) created by the LeukoStrat CDx *FLT3* Software. Click **OPEN** in the popup window and click **OK** in the import confirmation popup window.

11.16.10.1. If there is no match in the 3500xL or 3500xL Dx library for the assay name in the ABI file, click **Proceed** in the popup that appears:
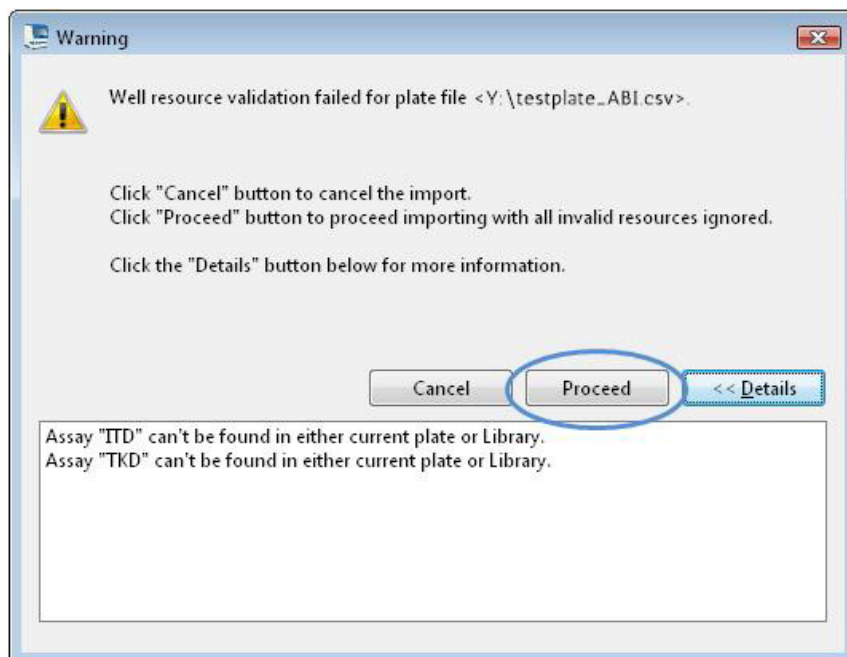
11.16.11. Click **OK** in the following popup window.
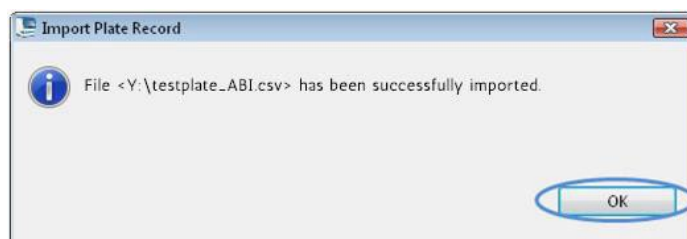
11.16.12. Once the import is complete, the Sample IDs populate the onscreen plate layout. Verify that the onscreen plate layout is correct by reviewing the Sample IDs on screen. If samples do not match the intended setup, a new ABI File needs to be created in the LeukoStrat CDx *FLT3* Software and re-imported into the ABI 3500xL or 3500xL Dx.

NOTE: Do not change the Sample IDs on the 3500xL or 3500xL Dx plate map. Doing so will result in an error.

11.16.13. Confirm the *Assays* programmed in the 3500xL or 3500xL Dx use the parameters listed in Table 6 for the **ITD CDx Assay** or **TKD CDx Assay**.
11.16.14. Assign the *Assay, Results Group*, and *File Name Convention* to all wells containing samples and controls, if necessary.

NOTE: *File Name Convention* must include the *Sample Name* as the first attribute.

11.16.15. Load the plate(s) on the 3500xL or 3500xL Dx.
11.16.16. Click **Link Plate for Run**. Operator may save changes to plate if prompted. If a second plate is to be run, repeat steps 11.16.2 through 11.16.15.

11.17. Run 3500xL or 3500xL Dx Genetic Analyzer

11.17.1. Check for bubbles in the POP-7 tubing. Remove bubbles, if needed.
11.17.2. Click **Start Run** to start the run on the 3500xL or 3500xL Dx.
11.17.3. After the run is complete, remove and discard the septa and discard the CE plate.

NOTE: In case of a connectivity error between the 3500xL or 3500xL Dx instrument and computer running Data Collection Software, follow the instrument manufacturer's troubleshooting instructions.

11.17.4. GeneMapper may be used to analyze files, proceed with section *Data Analysis with GeneMapper Software* (11.18).

11.18.   Data Analysis with GeneMapper Software

NOTE: It is a requirement to use *GeneMapper v6.0 with patch B02* (GM_PeakHeight_202110 patch for GeneMapper™Software Full v6.0) software. The patch should be applied on top of GeneMapper Software; contact the manufacturer to receive the patch and installation instructions.

NOTE: Do not override a size standard error for a well in the GeneMapper Software.

11.18.1.   Ensure use of *GeneMapper v6.0 with patch B02*.

11.18.1.1.   To confirm patch version, open *GeneMapper v6.0*. Navigate to the *Help Menu* tab and select **About GeneMapper Software 6**. View the *Build Id* on *About* pop-up.

11.18.2.   Open the *GeneMapper v6.0* software.

11.18.3.   Under the *File Menu*, choose **New Project** and select **Microsatellite**. Click **OK**. Return to the *File Menu* and choose **Add Samples to Project**.

11.18.4.   In the left panel, navigate to the data files in the 3500xL or 3500xL Dx data folder (designated by the *Results Group*) and click **Add to List** to transfer them to the right panel. Click the **Add** or **Add & Analyze** button.

11.18.5.   Ensure that the *Analysis Method* is set to a **Microsatellite** method and that the *Size Standard* is set to **GS600LIZ+Normalization** for all samples.

NOTE: If there are multiple assay types in one plate, the *Analysis Method* and *Size Standard* options can be set per injection for ease of workflow. *Injections* can be selected in the *Project* window.

11.18.6.   Ensure that the analysis method settings are set properly. Refer to Figure 3.

11.18.6.1.   Click **Analysis** and then **Analysis Method Editor** from the menu at the top of the screen.

11.18.6.2.   In the *Peak Detector* tab, ensure that the *Peak Detection Algorithm* is set to **Advanced**.

11.18.6.3.   Ensure under *Peak Amplitude Thresholds* that **100** is entered for the **B** (blue) and **G** (green), and **50** for the remaining **Y** (yellow), **R** (red), **P** (purple), and **O** (orange). Yellow and purple dye channels are not used in the LeukoStrat CDx *FLT3* Mutation Assay.

11.18.6.4.   Ensure that *Polynomial Degree* is set to **3** for ITD and set to **5** for TKD.

11.18.6.5.   Click **OK** at the bottom of the window.

NOTE: ITD and TKD specific Analysis Methods may be setup and used in GeneMapper by going to *Tools* and selecting the **GeneMapper Manager**. From the *Analysis Methods* tab, click the **New…** button and select **Microsatellite** as the analysis type. Click **OK**. Provide a *Name*, *Description,* and *Instrument* in the *General* tab, set up the *Peak Detector* tab as described above and in Figure 3, and leave the *Allele*, *Peak Quality* and *Quality Flags* tabs set to default *Microsatellite* settings. Select **Done** and the new Analysis Method is selectable.
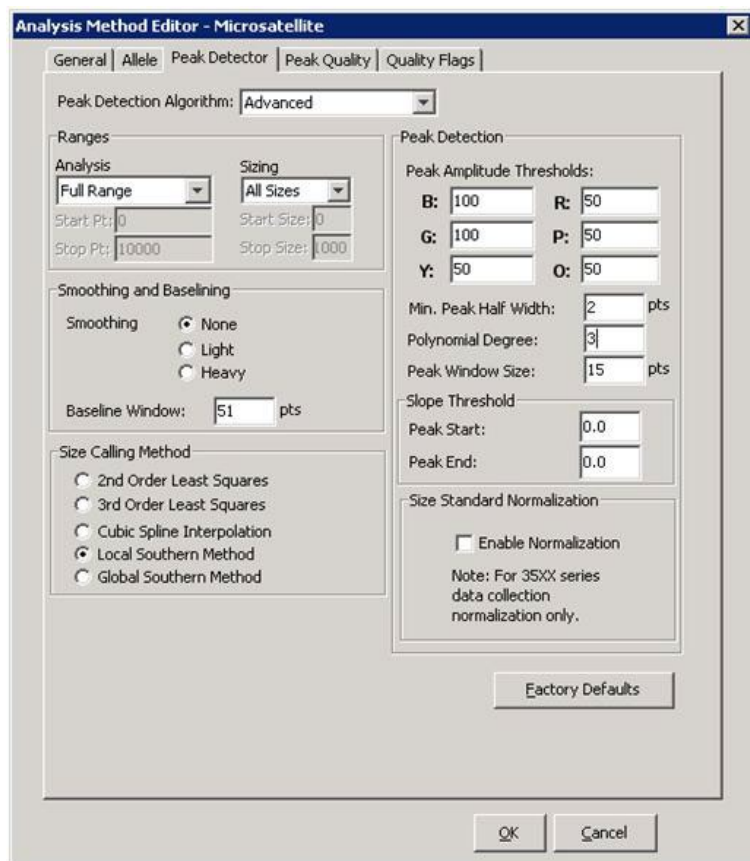
Figure 3. Analysis method settings for ITD. For TKD, settings are identical except that *Polynomial Degree* is set to **5**.

11.18.7.   Click the **green play button** to start analysis. Save the GeneMapper project with an appropriate name.

11.18.8.   In the GeneMapper software, highlight specimens and controls and click the **Display Plots** button.

11.18.8.1.   For ITD, ensure the **Sizing Table** icon is selected and that the blue, green, and red dyes are selected in the **Samples Plot** window.

11.18.8.2.   For TKD, ensure the **Sizing Table** icon is selected and that the blue and red dyes are selected.

11.18.9.   Ensure that the table below the electropherogram contains the following columns: **Dye/Sample Peak**, **Sample File Name**, **Size**, **Height**, and **Area**.

11.18.9.1.   If not, select **Tools** and then **Plot Settings…** from the **Samples Plot** menu.

11.18.9.2.   Select the **Sizing Table** tab and ensure that the following items have a green checkmark in the **Show** column: **Dye/Sample Peak**, **Sample File Name**, **Size**, **Height**, and **Area**.

11.18.9.3.   Click **OK**.

11.18.10.   To export the sizing table information, select **File** and then **Export Table** from the **Samples Plot** menu.

11.18.10.1.   Enter a file name and select the location to save the file.

11.18.10.2.   In the *Export File As* dropdown menu, select **Comma-separated values (.csv)**.

11.18.10.3.   Click **Export**.

NOTE: Do not edit the CSV file in any way.

11.19. Data Analysis with LeukoStrat CDx *FLT3* Software

11.19.1. Open the LeukoStrat CDx *FLT3* Software, accept the licensing agreement, and click on the *CDx Analysis* tab of the LeukoStrat CDx *FLT3* Software.

11.19.2. Click **Select Platemap File (*.livs)** and navigate to the LIVS file generated from the *PlateMapper Setup* tab.
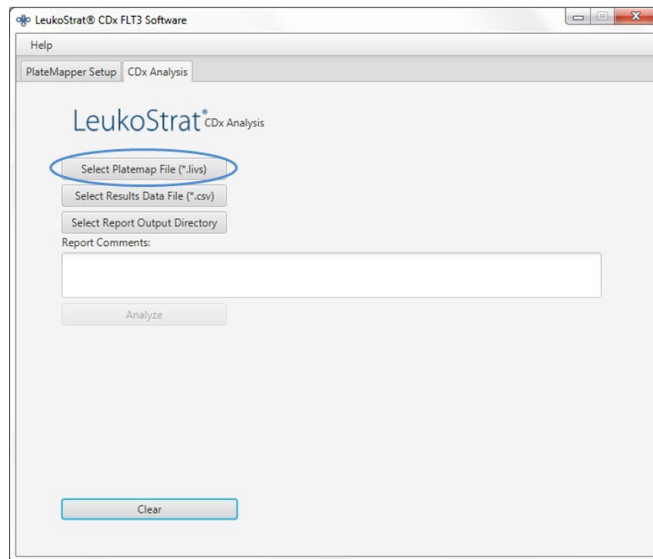
11.19.3. Click the **Select Results Data File (*.csv)** and select an exported CSV file from step 11.18.10 for analysis.
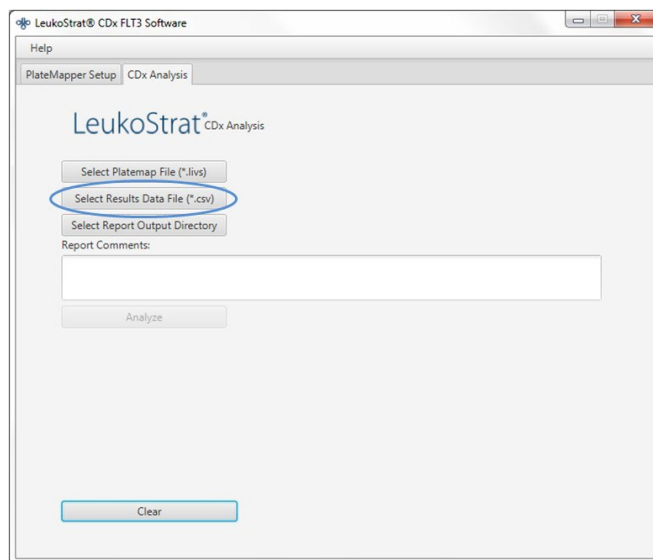
11.19.4. Click **Select Results Output Directory** and choose a destination folder for the results.
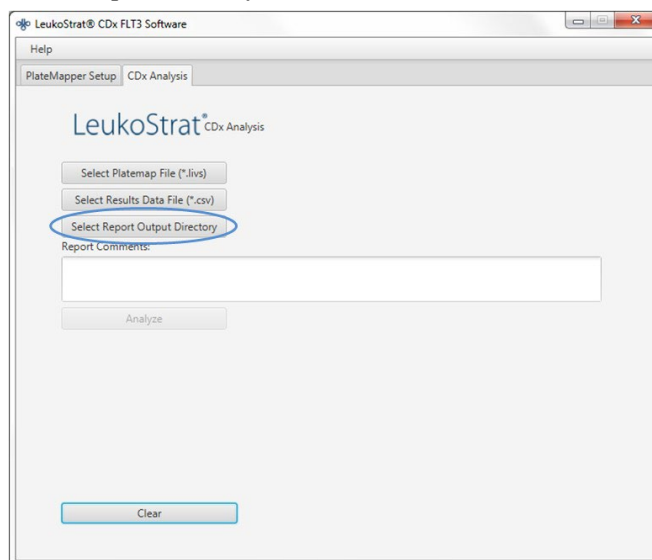
11.19.4.1. Additional comments about the run, samples or controls can be entered in the *Report Comments* field. These comments will appear in the *Run Report*.

NOTE: Once all files are selected, do not create or import a new *Plate Map* into the *PlateMapper Setup* tab prior to analyzing the current run/data. Alterations to the *PlateMapper Setup* tab prior to selecting the **Analyze** button will cause the report to display the incorrect *Plate Name*. Close the LeukoStrat CDx *FLT3* Software before switching between *PlateMapper Setup* tab and the *CDx Analysis* tab.

11.19.5. Once all three files are selected, the **Analyze** button will become selectable. Click **Analyze**, and three types of reports are generated in the destination folder – a *PDF Run Report, PDF Sample Report(s),* and *a CSV run export file* (see Figure 4, Figure 5, and Figure 6).

- The *Run Report* will contain a summary of results for all controls and samples.
- The *Sample Report* will contain results for the controls and details for the sample results.
- The *CSV run export file* will contain all of the run results in a spreadsheet format. IDs in the LeukoStrat CDx *FLT3* Software reports are the last 12 characters of the ID generated by the software.
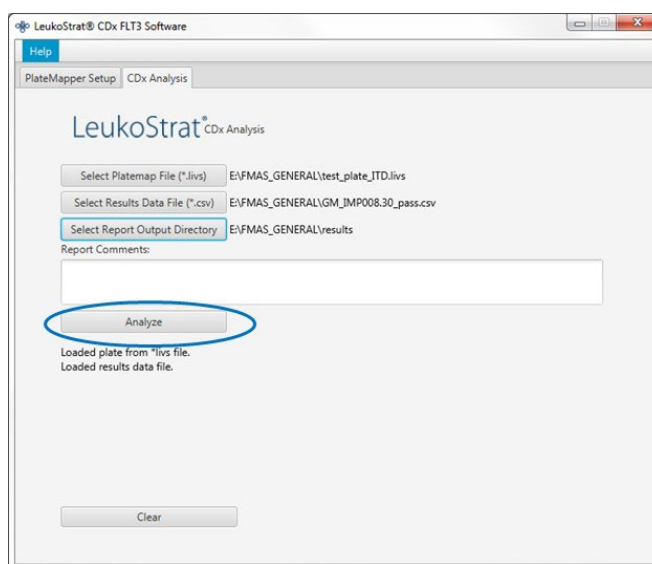
LeukoStrat® CDx *FLT3* Software

Run Report:

Run Information			
Run ID	fb170062-996c-4859-90c7-000000000001		
Plate ID	9dd67e4f-d8d0-4016-b72c-f7179eaae829	Assay	ITD
Plate Barcode	01234	Analysis Date	2022-12-02 10:49:49 AM
Plate Name	UnitTestPlate	Run Pass/Fail	Pass

Controls				
Type	Name	ID	Pass/Fail	Fail Detail
PC	PControl1_ITD_PC_H01	08277bd1d8e5	Pass	
NTC	NTCControl1_ITD_NTC_F01	4a6bf004cd22	Pass	
EC	ExtractionControl1_ITD_EC_E01	4e614e4d9b70	Pass	

Samples				
Sample Name	EC ID	Pos/Neg/Fail	Signal Ratio	Fail Detail
SampleA01_ITD_SAMPLE_A01	4e614e4d9b70	Positive	0.09	
SampleA02_ITD_SAMPLE_A02	4e614e4d9b70	Positive	0.07	
SampleA03_ITD_SAMPLE_A03	4e614e4d9b70	Positive	0.11	
SampleA04_ITD_SAMPLE_A04*	4e614e4d9b70	Negative	0.00	
SampleA05_ITD_SAMPLE_A05	4e614e4d9b70	Negative	0.00	
SampleA06_ITD_SAMPLE_A06	4e614e4d9b70	Negative	0.00	
SampleA07_ITD_SAMPLE_A07	4e614e4d9b70	Fail	N/A	IR91
SampleA08_ITD_SAMPLE_A08	4e614e4d9b70	Fail	N/A	IR91
SampleA09_ITD_SAMPLE_A09	4e614e4d9b70	Fail	N/A	IR91
SampleA10_ITD_SAMPLE_A10	4e614e4d9b70	Fail	N/A	IR91
SampleA11_ITD_SAMPLE_A11	4e614e4d9b70	Fail	N/A	IR91

Report Comments
N/A

* Indicates additional notes on Sample Report

LeukoStrat® CDx FLT3 Software
v1.1.x.IVD

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Figure 4. Example *Run Report.*

LeukoStrat®CDx *FLT3* Software

Sample Report:

Sample and Run Information				
Sample Name	SampleA01_ITD_SAMPLE_A01			
Sample ID	21c1a415-6fad-4f69-af8e-535ad212c275			
Plate ID	9dd67e4f-d8d0-4016-b72c-f7179eaae829		Assay	ITD
Plate Barcode	01234		Analysis Date	2022-12-02 10:49:49 AM
Plate Name	UnitTestPlate			
Run ID	fb170062-996c-4859-90c7-000000000001		Sample Pos/Neg/Fail	Positive

Controls				
Type	Name	ID	Pass/Fail	Fail Detail
PC	PControl1_ITD_PC_H01	08277bd1d8e5	Pass	
NTC	NTCControl1_ITD_NTC_F01	4a6bf004cd22	Pass	
EC	ExtractionControl1_ITD_EC_E01	4e614e4d9b70	Pass	

Sample				
Sample Name	EC ID	Pos/Neg/Fail	Signal Ratio	Fail Detail
SampleA01_ITD_SAMPLE_A01	4e614e4d9b70	Positive	0.09	

Sample Notes
N/A

Report Comments
N/A

LeukoStrat® CDx FLT3 Software
v1.1.x.IVD

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Figure 5. Example *Sample Report.*

Run ID	Assay	Run Result	Sample ID	Sample Type	EC ID	Sample Name	Sample Result	Signal Ratio	Sample Notes	Software Version
fb170062-996c-4859-90c7-000000000001	ITD	PASS	f7abf689-888c-4942-8202-08277bd1d8e5	PC		PControl1_ITD_PC_H01	POS	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	323e17c2-c7bf-4d57-9c86-4a6bf004cd22	NTC		NTCControl1_ITD_NTC_F01	UNSET	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	EC	08b5ee54-77a5-4159-a028-11f364c3c963	ExtractionControl1_ITD_EC_E01	NEG	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	21c1a415-6fad-4f69-af8e-535ad212c275	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA01_ITD_SAMPLE_A01	POS	0.09		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	29533bfb-b916-48c9-8ec1-e74444ca2be5	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA02_ITD_SAMPLE_A02	POS	0.07		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	5a6a01c9-d38d-48ea-a433-ea347e01b72b	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA03_ITD_SAMPLE_A03	POS	0.11		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	76a3ae2d-417d-4690-92f6-55521e593a6f	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA04_ITD_SAMPLE_A04	NEG	0	Validation Sample Note	v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	dd33cd5b-aa6f-473b-8565-386398d84912	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA05_ITD_SAMPLE_A05	NEG	0		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	8cf778b8-0353-49c7-bf93-cf842fc77b3a	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA06_ITD_SAMPLE_A06	NEG	0		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	55265e37-070c-4e9d-a418-95dd07099dbb	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA07_ITD_SAMPLE_A07	FAIL	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	d3c89c59-db82-4c39-8504-23153e174140	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA08_ITD_SAMPLE_A08	FAIL	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	b19bcd10-092c-47e1-bed1-fc0e30ed3dcf	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA09_ITD_SAMPLE_A09	FAIL	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	ac125670-78fe-42df-ab0e-1acae7f4a9c2	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA10_ITD_SAMPLE_A10	FAIL	N/A		v1.1.x.IVD
fb170062-996c-4859-90c7-000000000001	ITD	PASS	7a3b21f1-c898-424a-ab66-72c79c6c5c13	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA11_ITD_SAMPLE_A11	FAIL	N/A		v1.1.x.IVD

Figure 6.   Example *CSV run export file*

11.19.6.   Click **Clear** to reset all fields.
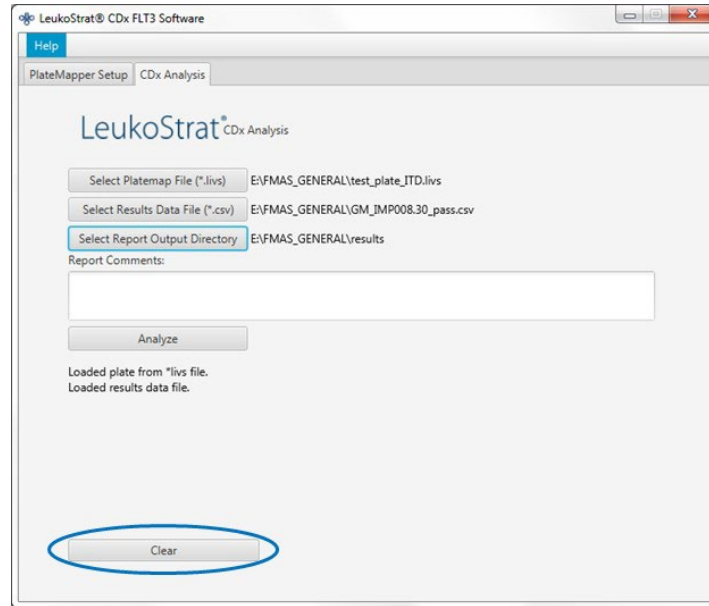
11.19.7.   If results are not obtained, verify all steps were completed correctly. Refer to Table 9 for troubleshooting data results errors. If further assistance is required, please contact Invivoscribe Technical Support at support@invivoscribe.com.

Table 9: Data Results Error Messages and Corrective Actions

Data Results Upload Error Message	Potential Cause(s)	Corrective Action(s)
• Unrecognized dye: <dye letter> [AD01]	Selection of unused dyes during GeneMapper software analysis step.	Ensure that only Red, Green, and Blue dyes are selected during the GeneMapper software analysis step.
• No red dye detected. Please make sure red dye is selected during previous signal analysis step. [AD02]	Did not select red dye during GeneMapper software analysis step	Make sure to select red dye during GeneMapper software analysis step.
• Unrecognized data results file format. [AD03]	GeneMapper software file is corrupted	Do not edit the GeneMapper software file in any way.
• Unable to load *.livs platemap file*; incorrect format. [AD04]	LIVS file is corrupted.	Do not edit the LIVS file in any way.
• Did not find run for runId <runId> [AD05] • Did not find sample for sample name <sampleName> [AD06]	Incorrect LIVS file selected, or LIVS file is corrupted.	Make sure to select the correct LIVS file associated with the experiment analyzed.

Table 9: Data Results Error Messages and Corrective Actions

Data Results Upload Error Message	Potential Cause(s)	Corrective Action(s)
• General error loading results data file; please contact technical support. [AD07]	Unknown error has occurred.	Contact Technical Support.
• String index out of range: -1	<Sample Name> was not the first attribute selected when setting up File Name Convention (step 11.16.14 this is done).	Repeat run beginning with step 11.12 *Capillary Electrophoresis Detection* - correct File Name Convention
• String index out of range: 15	CSV results file have been edited.	Repeat export of CSV results file from GeneMapper. Do not edit the CSV file in any way.

12. Quality Control

12.1. Run Validity

12.1.1.   The LeukoStrat CDx *FLT3* Software automatically evaluates the results.

12.1.2.   If the run status is *Fail*, all test results in the same run are invalid. Depending on the *Fail Detail*, the run must be repeated at different starting points within the assay (see section 14: *Retesting*).

12.2. Extraction Control and Sample Validity

12.2.1.   Within a valid run, individual samples may be invalid (*Fail*). If an Extraction Control does not meet validity criteria, all samples associated with that Extraction Control will be labeled *Fail*.

12.2.2.   Samples where all controls are valid may fail if they individually do not meet specifications. Depending on the *Fail Detail* in the LeukoStrat CDx *FLT3* Software, the sample(s) must be repeated at different starting points within the assay (see section 14: *Retesting*).

NOTE: If multiple failures of the same *Fail Detail* type are observed in a run, the retest strategy is different from isolated control or sample failures (see section 14: *Retesting*).

13. Interpretation of Results

13.1.   AML patients with a detectable *FLT3* ITD or TKD mutation at or above the clinical cut-off are indicated for gilteritinib fumarate therapy.

13.2.   AML patients with a detectable *FLT3* ITD mutation at or above the clinical cut-off are indicated for quizartinib hyrdochloride therapy. AML patients with only a detectable *FLT3* TKD mutation at or above the clinical cut-off are not indicated for quizartinib hydrochloride therapy.

13.3.   The mutant : wild-type signal ratio is calculated by the LeukoStrat CDx *FLT3* Software and automatically evaluated against the clinical 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.

13.4.   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 LeukoStrat CDx *FLT3* Software as 100; it is not intended to convey a ratio value.

13.5.   For determination of overall *FLT3* mutation status or whether gilteritinib fumarate is indicated:

13.5.1.   If the mutant : wild-type signal ratio for either ITD or TKD in a valid sample result is at or above the clinical cut- off of 0.05, the result will be interpreted and reported as **Positive** and gilteritinib fumarate is indicated.

13.5.2.   If the mutant : wild-type signal ratios for both ITD and TKD in a valid sample result are below the clinical cut-off of 0.05, the result will be interpreted and reported as **Negative** and gilteritinib fumarate is not indicated.

13.5.3.   The mutation status of a sample is defined by the rules included in Table 10.

Table 10: Determining Sample Mutation Status

Scenario	ITD Software Result	ITD Signal Ratio	TKD Software Result	TKD Signal Ratio	Final Assay Result
1	Positive	≥0.05	Positive	≥0.05	Positive
2	Negative	<0.05	Negative	<0.05	Negative
3	Invalid	N/A	Invalid	N/A	Invalid
4	Positive	≥0.05	Negative	<0.05	Positive
5	Negative	<0.05	Positive	≥0.05	Positive
6	Positive	≥0.05	Invalid	N/A	Positive
7	Negative	<0.05	Invalid	N/A	Invalid
8	Invalid	N/A	Positive	≥0.05	Positive
9	Invalid	N/A	Negative	<0.05	Invalid

13.6. For determination of whether quizartinib hydrochloride is indicated:

    13.6.1. If the mutant : wild-type signal ratio for ITD in a valid sample result is at or above the clinical cut-off of 0.05, the result will be interpreted and reported as **ITD Positive** and quizartinib hydrochloride is indicated.

    13.6.2. If the mutant : wild-type signal ratio for ITD in a valid sample result is below the clinical cut-off of 0.05, the result will be interpreted and reported as **ITD Negative** and quizartinib hydrochloride is not indicated.

    13.6.3. If the ITD result is invalid, the result will be interpreted and reported as **ITD Invalid** and quizartinib hydrochloride is not indicated.

13.7. Fail Detail codes are provided in the LeukoStrat CDx *FLT3* Software report; repeat the run or retest samples according to instructions in section 14: *Retesting*.

14. Retesting

14.1. Invalid Runs

    14.1.1. A run in which either Positive Control or No Template Control, or both, does not meet validity criteria is an invalid run. Repeat the run including all samples, Positive Control, all associated Extraction Controls, and No Template Control. ITD and TKD runs are independent of each other.

    14.1.2. Repeat the run according to Table 11 or Table 12, based on the assay and the specific Fail Detail(s) listed in the *Controls* section of the LeukoStrat CDx *FLT3* Software reports. The Fail Detail(s) listed for the failed Positive Control or No Template Control supersedes all Extraction Control and sample Fail Details.

14.2. Invalid Extraction Control within Valid Runs

    14.2.1. For Extraction Control failures within a valid run that may contain multiple Extraction Controls, retest all the failed Extraction Controls, associated samples, Positive Control, and No Template Control for the appropriate ITD or TKD run. Retest according to Table 11 or Table 12, based on the assay and the specific Fail Detail(s) listed in the *Controls* section of the LeukoStrat CDx *FLT3* Software reports. The Fail Detail(s) listed for the failed Extraction Control supersedes all sample Fail Details.

14.3. Invalid Samples within Valid Runs

    14.3.1. For sample failures within a valid run, retest the sample(s), Positive Control, Extraction Control(s) associated with the failed sample(s), and No Template Control for the appropriate ITD or TKD run. Retest according to Table 11 or Table 12, based on the assay and the specific Fail Detail(s) listed in the *Samples* section of the LeukoStrat CDx *FLT3* Software reports. Retesting of a sample must include retesting of the associated Extraction Control.

14.4. Fail Details and Retesting

    14.4.1. Table 11 and Table 12 summarize retesting based on the Fail Detail by sample type for ITD and TKD, respectively. Consult Table 13. for the retest codes listed in Table 11 and Table 12.

    14.4.2. The hierarchy of retesting is:

        1) Invalid ITD or TKD Positive Control (PC) or No Template Control (NTC) (see section 14.1)

        2) Invalid Extraction Control (EC) within a valid run (see section 14.2)

        3) Invalid samples within a valid run (see section 14.3)

Figure 7 diagrams the hierarchy of retesting.

14.4.3. If more than one failure within a single sample or control has occurred, perform the retest that returns the operator to the step closest to the beginning of the assay procedure.

14.4.3.1. If the same Fail Detail occurs in the same control/sample, proceed to the next Retest Starting Point, if one is listed. If the same Fail Detail occurs again after all the troubleshooting actions have been completed, the control/sample result is invalid.

14.4.3.2. If the results of the retest are a different failure mode than the initial results, follow the troubleshooting action described for the new retest failure mode.

NOTE: No more than four retests are allowed for a single control/sample.

14.4.4. Invalid samples are assessed independently; thus, if multiple samples with different Fail Details for each sample are identified within a single run, perform the retest that is appropriate for each sample.

NOTE: If further assistance is required, please contact Invivoscribe Technical Support at support@invivoscribe.com.
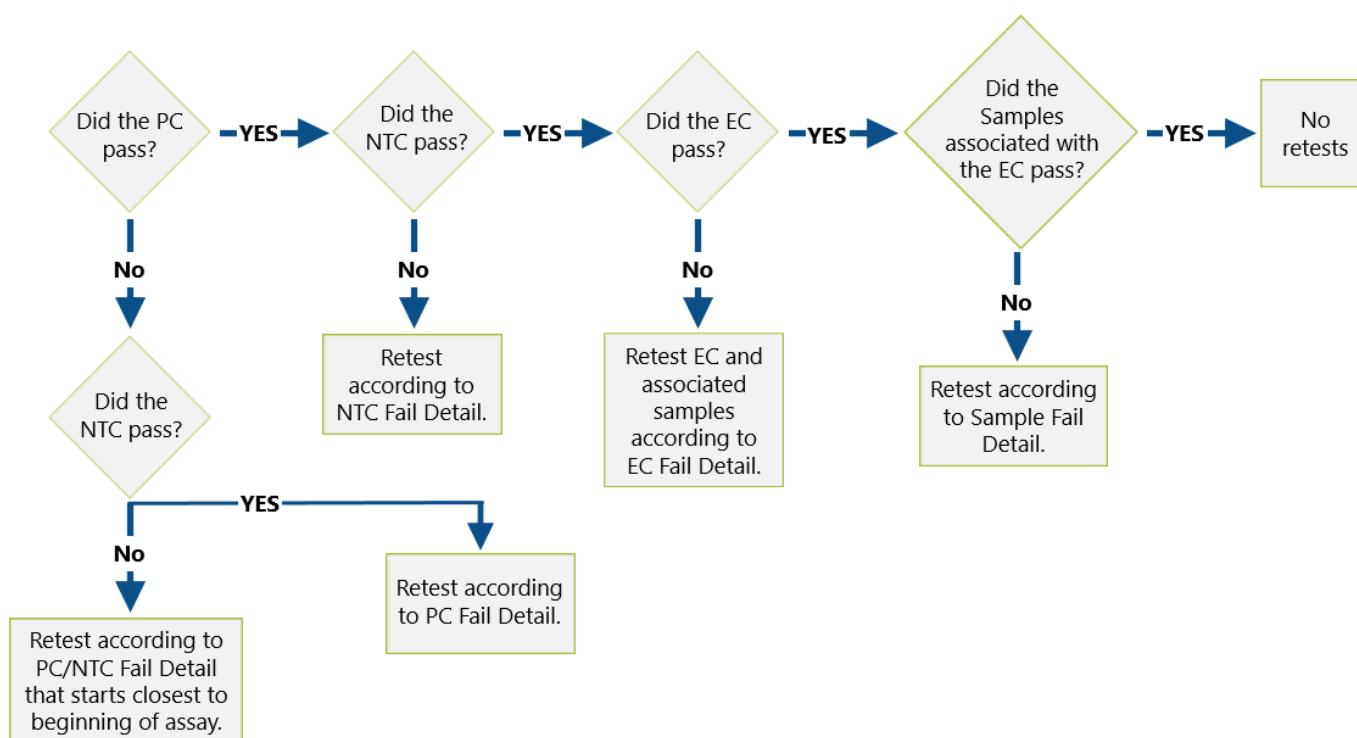
Figure 7. Retest hierarchy diagram

Table 11: Retesting, ITD Controls and Samples

ITD Fail Detail	Controls			Samples	
	PC	NTC	EC	Pos Sample	Neg Sample
IR05: Sample or Control Failed.	Amp		Amp		Quant/Proc
IR06: Sample or Control Failed.	Amp				Quant/Proc
IR07: Sample Failed.				CE-DS	CE-DS
IR09: Sample or Control Failed.	CE	CE	CE	CE/Proc	CE/Proc
IR12: Sample or Control Failed.	Amp		Q-Amp	Quant/Proc	Quant/Proc
IR13: Control Failed.	CE				
IR20: Control Failed.				Ctrl	Ctrl

Table 11: Retesting, ITD Controls and Samples

ITD Fail Detail	Controls			Samples	
	PC	NTC	EC	Pos Sample	Neg Sample
IR21: Control Failed.				Ctrl	Ctrl
IR31: Control Failed.	Amp				
IR32: Control Failed.	CE/Amp				
IR33: Control Failed.	CE/Amp				
IR34: Control Failed.	Amp				
IR40: Control Failed.		Amp			
IR51: Control Failed.			Q-Amp		
IR52: Control Failed.			CE/Q-Amp		
IR53: Control Failed.				Ctrl	Ctrl
IR70: Sample Failed.				CE/Proc	
IR80: Sample Failed.					Quant/Proc
IR91: Sample or Control Failed.	CE	CE	CE	CE	CE

Table 12: Retesting, TKD Controls and Samples

TKD Fail Detail	Controls			Samples	
	PC	NTC	EC	Pos Sample	Neg Sample
TR07: Sample or Control Failed.	Dig		Dig		Dig/Proc
TR09: Sample or Control Failed.	CE	CE	CE	CE/Proc	CE/Proc
TR12: Sample or Control Failed.	Amp		Q-Amp	Quant/Proc	Quant/Proc
TR20: Control Failed.				Ctrl	Ctrl
TR21: Control Failed.				Ctrl	Ctrl
TR30: Control Failed.	Xtalk/Amp				
TR31: Control Failed.	CE/Amp				
TR32: Control Failed.	CE/Amp				
TR33: Control Failed.	Amp				
TR40: Control Failed.		Amp			
TR50: Control Failed.			Xtalk/Q-Amp		
TR51: Control Failed.			CE/Q-Amp		
TR52: Control Failed.			Dig		
TR53: Control Failed.				Ctrl	Ctrl
TR70: Sample Failed.				Xtalk/Quant/Pro	
TR71: Sample Failed.				CE/Proc	
TR72: Sample Failed.				Dig/Proc	
TR80: Sample Failed.					Xtalk/Quant/Proc
TR81: Sample Failed.					Quant/Proc
TR93: Sample or Control Failed.	CE	CE	CE	CE	CE

Table 13: Retest codes

Retest Code	Description	Retest Starting Point
Amp	Repeat beginning at amplification, using previously made DNA sample dilutions.	11.10. *Amplification*
CE	Repeat beginning at capillary electrophoresis. Make a new CE plate with fresh amplicon (from the stored ITD PCR or TKD digestion plate) ensuring the Positive Control, No Template Control, and associated Extraction Control(s) are also present on the plate with a failed sample.	11.12. *Capillary Electrophoresis Detection*
CE/Amp CE/Q-Amp	Retest following the instructions for the CE retest code. If the retest results yield the same Fail Detail, retest again following the instructions for Amp or Q-Amp, as prescribed.	11.12. *Capillary Electrophoresis Detection* 11.9. *Quantification and Dilution of DNA* 11.10. *Amplification*
CE/Proc	Retest following the instructions for the CE retest code. If the retest results yield the same Fail Detail, reprocess the sample beginning from peripheral blood or bone marrow aspirate.	11.12. *Capillary Electrophoresis Detection* 11.2. *Sample Processing Preparation*
CE-DS	Repeat beginning at capillary electrophoresis. Make a new CE plate with fresh amplicon (from the stored ITD PCR plate) ensuring the Positive Control, No Template Control, and the associated Extraction Control(s) are also present on the plate (see section 14.6 *Dye Shift*). If the same Fail Detail (IR07) occurs a second time, report the sample as Invalid.	11.12. *Capillary Electrophoresis Detection*
Ctrl	Retest following the instructions for the control that failed.	Varies
Dig	Repeat beginning at digestion, using fresh amplicon (from stored TKD PCR plate) of the sample(s), associated Extraction Control(s) and controls.	11.11. *Restriction Digest (TKD Mutation Only)*
Dig/Proc	Retest following the instructions for the Dig retest code. If the retest results yield the same Fail Detail, reprocess the sample beginning from peripheral blood or bone marrow aspirate.	11.11. *Restriction Digest (TKD Mutation Only)* 11.2. *Sample Processing Preparation*
Q-Amp	Repeat beginning at quantification of Extraction Control(s), using previously made DNA sample dilutions.	11.9. *Quantification and Dilution of DNA* 11.10. *Amplification* (Samples)
Quant	Repeat beginning at quantification of all sample(s) and associated Extraction Control(s).	11.9. *Quantification and Dilution of DNA* 11.10. *Amplification* (Samples)
Quant/Proc	Retest following the instructions for the Quant retest code. If the retest results yield the same Fail Detail, reprocess the sample beginning from peripheral blood or bone marrow aspirate.	11.9. *Quantification and Dilution of DNA* 11.2. *Sample Processing Preparation*
Xtalk/Amp Xtalk/Q-Amp Xtalk/Quant/Proc	Make a new CE plate such that all samples are separated by 5 empty capillaries (*i.e.* only load samples in wells A01, C01, E01, and G01 for injection 1. Load samples in the equivalent wells for the remaining injections). If the retest results yield the same Fail Detail, retest following the instructions for Amp, Q-Amp, or Quant, as prescribed. If the Quant retest results yield the same Fail Detail, reprocess the sample beginning from peripheral blood or bone marrow aspirate.	11.12. *Capillary Electrophoresis Detection* 11.9. *Quantification and Dilution of DNA* (Extraction Control(s)) 11.10. *Amplification* (Samples, PC) 11.2. *Sample Processing Preparation*

14.5. Multiple Failures within a Run

14.5.1. In contrast to isolated invalid sample or Control Results, some Fail Details can be observed in several to all reaction wells. When this type of failure occurs, repeat the run including all samples, Positive Control, all associated Extraction Controls, and No Template Control according to Table 14; retest codes are listed in Table 15.

14.5.2. Additional troubleshooting actions may include the following items:

14.5.2.1.   Open the CSV file to confirm it contains results for all sample and control wells that have an associated 3500xL or 3500xL Dx FSA file.

14.5.2.2.   In the CSV file, ensure the proper columns are present, the peak thresholds are correct (*i.e.* no peaks less than 100 in Blue and Green or less than 50 in Red), and that the columns are populated with non-zero numbers.

Table 14: Retesting, Multiple Failures within a Run

Sample Type	Fail Detail	Retest Code
ITD PC	IR31	Amp
ITD NTC	IR40	
ITD EC	IR51	
TKD PC	TR30	
TKD NTC	TR40	
TKD EC	TR50	
ITD PC	IR33	CE/Amp
ITD Sample	IR70	
TKD PC	TR32	
TKD Sample	TR71	
ITD PC	IR32	
ITD EC	IR52	
ITD Sample	IR80	
TKD PC	TR31	
TKD EC	TR51	
TKD Sample	TR81	
All ITD within an injection	IR91	CE-SS
All TKD within an injection	TR93	
All ITD within a run	IR04	GM
All TKD within a run	TR04	

Table 15: Retest Codes, Multiple Failure Retesting

Retest Code	Description	Retest Starting Point
Amp	Repeat beginning at amplification, using previously made test sample DNA dilutions. Ensure all tubes are vortexed per instructions and that Taq was added.	11.10. *Amplification*
CE	Repeat beginning at capillary electrophoresis. Make a new CE plate with fresh amplicon (from the stored ITD PCR or TKD digestion plate) and fresh size standard solution. Ensure the Positive Control, No Template Control, and associated Extraction Control(s) are also present on the plate with the failed sample.	11.12. *Capillary Electrophoresis Detection*
GM	Repeat creation of export data files from GeneMapper software, ensuring the threshold(s) are set to 100 RFU for blue and green.	11.18 *Data Analysis with GeneMapper Software*
CE/Amp	Retest following the instructions for the CE retest code. If the retest results yield the same Fail Detail, retest again following the instructions for Amp.	11.12. *Capillary Electrophoresis Detection* 11.10. *Amplification*
CE-SS	Repeat beginning at capillary electrophoresis, using a new preparation of size standard solution.	11.12. *Capillary Electrophoresis Detection*

14.6.   Dye Shift

In rare instances with some large ITD inserts, the LeukoStrat CDx *FLT3* Software may misidentify confirmation of a mutant peak. To confirm the dye shift, repeat capillary electrophoresis by making a new CE plate with fresh amplicon from the stored ITD PCR plate.

15. Limitations of Procedure

15.1.   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.

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

15.3.   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.

  ▪ ITD insertions sized between 3 bp and 30 bp will be reported as ITD mutations.
  ▪ ITD insertions sized between 279 bp and 323 bp will be reported as ITD mutations.
  ▪ ITD insertions sized greater than 323 bp are not detectable by the assay.

15.4.   This assay may not detect *FLT3* mutations that present below the sensitivity level of the assay.

  15.4.1.   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.

  15.4.2.   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.

  15.4.3.   For TKD mutations that modify the EcoRV site, an allelic ratio of 0.18 will yield a positive LeukoStrat CDx *FLT3* Mutation Assay result.

15.5.   This assay exhibits different rates of signal ratio variability based on the *FLT3* mutation type, presented below.

  15.5.1.   For ITD insertions sized 21 bp to 90 bp, inclusive, the signal ratio variability ranges from 4.4% to 8.5%.

  15.5.2.   For ITD insertions sized 217 bp, the signal ratio variability ranges from 26.9% to 27.2%.

  15.5.3.   For TKD mutations that modify the EcoRV site, the signal ratio variability ranges from 4.2% to 5.9%.

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

15.7.   The clinical performance of the test using data from the clinical accuracy study was determined to be:

  15.7.1.   Diagnostic Sensitivity: 1
  15.7.2.   Diagnostic Specificity: 0.92
  15.7.3.   Positive Likelihood Ratio: 12.5
  15.7.4.   Negative Likelihood Ratio: 0

15.8.   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.

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

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

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

16. Expected Values

16.1.   Expected Size of Amplified Products

  16.1.1.   The amplicon sizes listed were determined using a 3500xL and 3500xL Dx instrument (Table 16).

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

Table 16: Expected Amplicon Sizes

Master Mix	Part #	Target	Dye Channel	Control DNA	Product Size in Nucleotides
FLT3 ITD	R0880060 R0880080	Exon 14 and 15	**Blue** & **Green**	**Valid Size Range** *FLT3* ITD Positive Control DNA *FLT3* Extraction Control DNA	**326-650** 327±1, 357±1 327±1
FLT3 TKD	R0880070 R0880080	Exon 20	**Blue**	**Valid Size Range** *FLT3* TKD Positive Control DNA *FLT3* Extraction Control DNA	**78-80, 124-128** 79±1, 127±1 79±1, 127±1[a]

[a]**Note:**   A small 127 bp product peak may or may not be present in the extraction control.

17. Non-Clinical Performance Evaluation

17.1. Analytical Sensitivity – Limit of Blank (LoB)

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.

17.2. Analytical Sensitivity

17.2.1. 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 four ITD insert sizes: 21 bp insert, 30 bp insert, 126 bp insert and a 279 bp insert. An additional cell line containing the D835 mutation was also assessed. DNA was diluted to 5 ng/µL, 10 ng/µL and 15 ng/µL and tested at multiple 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 Table 17. 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 moderate positive (MP). These linear range samples were tested in the LeukoStrat CDx *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 equal to or above the clinical cut-off in ≥95% of replicates (Above Cut-off).

Table 17: 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	0.01	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)	19 (95.0)	Cut-off Above Cut-off
		LP	0.07	0.08	0.152	20	20 (100.0)	20 (100.0)	-
		MP	0.13	0.15	0.269	20	20 (100.0)	20 (100.0)	-
ITD CS1	ITD 24 bp	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)	20 (100.0)	Cut-off Above Cut-off
		LP	0.08	0.08	0.165	20	19 (95.0)	19 (95.0)	-
		MP	0.13	0.13	0.257	20	20 (100.0)	20 (100.0)	-
ITS-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	-

Table 17: 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
		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	-
		HN	0.02	0.02	0.147	20	15 (75.0)	0	-
		CO	0.04	0.04	0.276	20	20 (100.0)	9 (45.0)	Analytical LoD 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
True Neg TKD	None	TN	N/A	0	0	20	0	0	N/A

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

17.2.2.   The LeukoStrat CDx *FLT3* Mutation Assay is capable of detecting the following mutant/wild-type allelic ratios above the clinical cut-off of the following mutation types:

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

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

17.2.2.3.   For ITD insertions sized at 217 bp, an allelic ratio of 0.539 was detected above the cut-off SR in more than 95% of samples. The SR %CV for these samples was 25.6%.

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

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

17.2.2.6.   Conversion of AR values to % Mutant is shown in the following table.

Table 18: Analytical Sensitivity Allelic Ratio and % Mutant

Sample ID	Mutation	Mutation Classification	Above Cut-off 95% SR≥ 0.05		
			AR	SR	%Mut
TKD CS1	TKD I836	TKD I836 Deletion	0.144	0.07	12.6
TKD CS2	TKD D835	TKD D835 Substitution	0.089	0.05	8.2
ITD CS1	ITD 24 bp	Small ITD Insert <30 bp	0.107	0.05	9.7
ITD CS2	ITD 66 bp	Medium ITD Insert 30-100 bp	0.189	0.08	15.9
ITD CS3	ITD 217 bp	Large ITD Insert ~200 bp	0.539	0.08	35.0

17.3.   Precision

17.3.1.   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 21 bp to 126 bp and TKD mutation samples. The 10 replicates were tested in batches of two, a total of five separate times.

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

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

17.4. Operator-to-Operator Reproducibility (cell-lines)

17.4.1. Samples consisted of ITD cell lines containing inserts of 21 bp, 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%.

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

17.4.3. For ITD mutation samples up to and including 30 bp inserts, the SR %CV ranged from 6.6% to 9.4%.

17.4.4. For ITD mutation samples at 126 bp inserts, the SR %CV ranged from 9.0% to 13.3%.

17.5. Operator-to-Operator Reproducibility (clinical samples)

17.5.1. In a second study, precision was assessed using clinical DNA samples from 7 clinical samples (5 PB and 2 BM) with ITD lengths of 21 bp, 24 bp, 66 bp, 90 bp and 217 bp, TKD D835 substitution, TKD I836 deletion, and 8 (4 PB and 4 BM) *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.

17.5.2. The total %CV of all mutation types and levels are shown in Table 19 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%. 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 19: Components of Variance per Mutation Type and Dilution Level

Sample ID	Mut Type	Dilution Level	Mean SR	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	HN	0.032	0.001 (0.90%)	0.002 (7.20%)	0.008 (91.90%)	0.009	27.2

Table 19: 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
	217 bp	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

17.6. Lot-to-Lot and Instrument-to-Instrument Reproducibility

17.6.1. The lot-to-lot and instrument-to-instrument reproducibility was determined by a single operator testing the same set of samples using three lots of reagents on three sets of instruments. Cell line samples consisted of ITD samples containing inserts ranging in size from 21 bp to 126 bp and TKD mutation samples.

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

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

17.7. Interfering Substances – Exogenous

17.7.1. 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.

17.8. Interfering Substances – Endogenous

17.8.1. 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 lipids/triglycerides, hemoglobin, protein and bilirubin.

17.9. Interfering Substances – Treatment Drugs

17.9.1. 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 cytarabine and daunorubicin.

17.10. Carryover and Cross Contamination

17.10.1. 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:

17.10.1.1. Carryover / Cross Contamination detected was 0%.

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

17.11. DNA Input

17.11.1. 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 1 279 bp ITD (11 replicates)

17.11.2. Acceptance criteria were met for 30 bp ITD, 126 bp ITD and D835 TKD cell line samples: 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%.

17.11.3. 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.

17.12. Validation of EDTA Blood Collection Tubes

17.12.1. The purpose of this study was to validate EDTA Blood collection tubes. This study used contrived samples consisting of ITD cell lines containing inserts of 21 bp, 126 bp, and 279 bp and the D835 TKD Mutation cell line spiked into

peripheral blood collected in Na-Heparin or EDTA. Samples represented high negative, low positive (near cut-off), and moderate positive mutant : wild-type SRs. Peripheral blood alone was used as the true negative samples.

17.12.2. Low positive and moderate positive samples resulted in 100% positive replicates in both EDTA and Na-Heparin. High negative and true negative samples resulted in 100% negative replicates in both EDTA and Na-Heparin. Therefore the acceptance criteria were met.

17.12.3. All validation acceptance criteria were met and EDTA Blood Collection Tubes are validated for use with the LeukoStrat CDx *FLT3* Mutation Assay.

17.13. Density Gradient Media Validation

17.13.1. The purpose of this study was to validate the use of any density gradient media (with a 1.077 g/mL density) in the LeukoStrat CDx *FLT3* Mutation Assay. Cell lines (21 bp insert, 279 bp insert, and TKD D835) were mixed with healthy peripheral blood at three low mutant cell fractions per cell line (resulting in nine panel members). Healthy peripheral blood was also tested as the *FLT3* negative sample (resulting in one panel member). Mononuclear cells were isolated from two replicates using three density gradient media (DGM) manufacturers by two operators on two days yielding a total of eight isolation replicates per panel member per density gradient media.

17.13.2. The percent of overall positive calls of two additional DGM manufacturers (DGM2 and DGM3) was compared to the DGM that was initially validated for use with the LeukoStrat CDx *FLT3* Mutation Assay (DGM1). DGM1 had 37.5% positive calls across the 10 panel members. DGM2 had 35% and DGM3 had 36.3% positive calls; therefore meeting the requirement of the overall positive calls being within 10% of DGM1 (2.5% and 1.2%, respectively).

17.13.3. All study acceptance criteria were met which validates the use of any 1.077 g/mL density gradient media in the LeukoStrat CDx *FLT3* Mutation Assay.

17.14. NEBuffer 3.1 Validation

17.14.1. This study was designed to provide objective evidence that NEBuffer 3.1 can be used in the LeukoStrat CDx *FLT3* Mutation Assay instead of NEBuffer 3 and BSA. Five positive TKD D835 substitution, 5 positive TKD I836 deletion, one TKD D835 cut-off, and eight TKD negative DNA samples were tested with one lot of NEBuffer 3 and BSA and three lots of NEBuffer 3.1. Three replicates per NEBuffer were tested yielding a total of 12 replicates per sample.

17.14.2. All positive samples, by NEBuffer 3 and BSA, were also positive by NEBuffer 3.1. All negative samples, by NEBuffer 3 and BSA, were negative by NEBuffer 3.1 yielding 100% agreement between the NEBuffer types. The signal ratio percent difference between the NEBuffer types ranged from -4% to 5% for the positive and cut-off samples. The signal ratio %CV range for NEBuffer 3 was 0 to 12.4% and for NEBuffer 3.1 was 0 to 10.7%.

17.14.3. All study acceptance criteria were met which validates the use of NEBuffer 3.1 in the LeukoStrat CDx *FLT3* Mutation Assay.

17.15. Equivalency: NEBuffer r3.1 vs NEBuffer 3.1

17.15.1. The study was to provide objective evidence that NEBuffer r3.1 is equivalent to NEBuffer 3.1 for LeukoStrat CDx *FLT3* Mutation Assay. NEBuffer (3.1 or r3.1) is used with the restriction endonuclease enzyme EcoRV to digest TKD amplicons in order to detect two TKD mutations (D835 and I836) by LeukoStrat CDx *FLT3* Mutation Assay. The only difference between NEBuffer 3.1 and NEBuffer r3.1 is that the bovine serum albumin within NEBuffer 3.1 is replaced by the recombinant albumin within NEBuffer r3.1. The study was designed to test 8 TKD positive clinical DNA samples (containing at least one sample with I836 mutation) and 8 TKD negative clinical DNA samples in triplicates using 3 lots of NEBuffer r3.1 to compare with 1 lot of NEBuffer 3.1.

17.15.2. There was 100% agreement between NEBuffer r3.1 and 3.1 for all samples. All TKD positive samples were correctly called as positive and all TKD negative samples were correctly called as negative.

17.15.3. NEBuffer r3.1 has been validated for use with the LeukoStrat® CDx *FLT3* Mutation Assay as all acceptance criteria were met.

17.16. Multiple Site Precision and Reproducibility

17.16.1. The purpose of this study was to determine if the LeukoStrat CDx *FLT3* Mutation Assay performed as intended when tested at three separate sites. The samples were contrived using cell line DNA with a 126 bp insert and a 279 bp insert, ITD clinical DNA with a 6 bp insert, 69 bp insert, and 193 bp insert, TKD clinical DNA with a TKD D835 substitution and a TKD I836 deletion, and *FLT3* negative clinical DNA. All clinical samples with a mutation were tested at three signal ratio levels: high negative, low positive, and moderate positive (resulting in 15 panel members). Two panel members were made from the clinical negative DNA and the cell line DNA samples were tested at two signal ratio levels: high negative and low positive (resulting in four panel members). A total of 21 panel members were tested by each site.

17.16.2. Two operators per site, on two non-consecutive days per operator, tested three replicates per panel member, alternating between two of three kit lots per site. Each site tested a total of 24 replicates per panel member for a total of 72 replicates per panel member for this study.

17.16.3. The signal ratio %CV for positive panel members (excluding long ITD insert panel members) ranged from 3.8% to 13.4% for all three sites combined (Table 20) and from 3.3% to 19.8% for each site individually (less than the required 25% CV).

Table 20: Components of Variance per Low and Moderate Panel Member

PM Type	SR Level	N	Overall Mean	Site/Instrument		Operator		Day/Run		Kit Lot		Random Error		Total Variability	
				SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Short 6 bp ITD	LP	72	0.097	0.000	0.0	0.001	1.5	0.001	1.0	0.002	2.1	0.005	5.0	0.006	5.7
	MP	72	0.171	0.000	0.0	0.003	1.6	0.002	1.2	0.000	0.0	0.006	3.2	0.007	3.8
Medium 69 bp ITD	LP	72	0.104	0.000	0.0	0.000	0.0	0.002	1.8	0.000	0.0	0.007	6.3	0.007	6.6
	MP	72	0.184	0.003	1.8	0.006	3.3	0.000	0.0	0.008	4.2	0.021	11.2	0.023	12.6
		70*	0.183	0.000	0.0	0.004	2.2	0.002	1.3	0.006	3.1	0.008	4.5	0.011	6.0
Medium 126 bp ITD	LP	72	0.095	0.000	0.0	0.002	2.0	0.006	5.8	0.001	1.1	0.008	8.1	0.010	10.2
Long 192 bp ITD	LP	72	0.084	0.000	0.0	0.000	0.0	0.012	14.2	0.001	1.4	0.012	14.6	0.017	20.4
	MP	72	0.173	0.000	0.0	0.000	0.0	0.016	9.5	0.010	5.7	0.011	6.3	0.022	12.7
Long 279 bp ITD	LP	72	0.073	0.000	0.0	0.010	13.9	0.007	9.6	0.010	13.8	0.018	24.3	0.024	32.6
TKD D835	LP	72	0.095	0.002	2.3	0.004	4.1	0.004	4.3	0.000	0.0	0.007	7.5	0.009	9.9
	MP	72	0.164	0.007	4.2	0.001	0.7	0.000	0.0	0.004	2.4	0.021	12.5	0.022	13.4
		71*	0.162	0.004	2.4	0.000	0.0	0.004	2.3	0.000	0.0	0.007	4.3	0.009	5.4
TKD I836	LP	72	0.083	0.002	2.1	0.000	0.0	0.003	4.0	0.001	0.7	0.004	4.3	0.005	6.2
	MP	72	0.153	0.004	2.4	0.000	0.0	0.003	2.3	0.002	1.5	0.006	4.0	0.008	5.4

*Outliers were removed: two Moderate Positive Medium 69 bp ITD and one Moderate Positive TKD D835

17.16.4. The lower limit of the Clopper-Pearson 95% two-sided confidence intervals for the positive and negative percent agreements (excluding long ITD insert panel members) for all three sites combined was ≥ 95.0% and 90.3%, respectively, exceeding the required 90% criterion.

17.16.5. All study acceptance criteria were met which validates a distributable version of the LeukoStrat CDx *FLT3* Mutation Assay for use by additional sites.

17.17. Peripheral Blood vs Bone Marrow Specimen Equivalency

17.17.1. The LeukoStrat CDx *FLT3* Mutation Assay is intended to detect mutations in genomic DNA (gDNA) isolated from either peripheral blood (PB) or bone marrow (BM) aspirates. A study was conducted to provide objective evidence that gDNA isolated from paired BM and PB yield concordant results from both specimen types.

17.17.2. BM and PB paired specimens (from the same subject and collection date) were prospectively collected from Clinical Collection Sites to support analytical validation studies. Paired specimens were also collected as part of the Astellas 2215-CL-0301 study. The sample set was comprised of 95 pairs: 62 from Clinical Collection Sites and 33 pairs from Astellas 2215-CL-0301 study. The average positive agreement (APA) and the average negative percent agreement (ANA) were calculated using the two PPAs and NPAs weighted by the corresponding marginal totals. In addition, the 95% confidence intervals for APA and ANA were calculated using the non-parametric bootstrapping percentile method.

17.17.3. Table 21 shows the concordance table between the PB and the BM results on overall *FLT3* mutation status. As shown in the table below, 94 of the 95 patients were concordant between PB and BM and only one pair yielded a discordant result. This result was associated with BM sample result at the clinical cut-off (SR = 0.05).

Table 21: Concordance between Peripheral Blood and Bone Marrow – Overall *FLT3* Mutation Status

PB	BM		Total
	BM+	BM-	
PB+	35	0	35
PB-	1	59	60
Total	36	59	95

17.17.4. Table 22 shows the agreement between BM and PB using BM and PB as a reference. The point estimates of NPA, PPA and OPA were all above 97%. The OPA lower bound of the 95% confidence interval was above 94% demonstrating the agreement between the BM and PB specimen types.

Table 22: Agreement between Peripheral Blood and Bone Marrow – Overall *FLT3* Mutation Status

Agreement	Percent Agreement (N)	95% CI[1]
PPA|BM	97.2% (35 / 36)	(85.5%, 99.9%)
PPA|PB	100% (35 / 35)	(90.0%, 100%)
NPA|BM	100% (59 / 59)	(93.9%, 100%)
NPA|PB	98.3% (59 / 60)	(91.1%, 100%)
OPA	98.9% (94 / 95)	(94.3%, 100%)

[1]The 95% CI is calculated using Exact (Clopper Pearson) method.

17.17.5. Table 23 shows the average positive percent agreement (APA) and the average negative percent agreement (ANA) between CDx results obtained in peripheral blood and bone marrow. The APA (ANA) was calculated as the weighted average of the PPA (NPA) using PB as reference and the PPA (NPA) using BM as reference. The point estimate of APA and ANA are 98.6% and 99.2%. The lower bounds of the 95% confidence intervals are above 95% for APA and ANA, demonstrating agreement between results for PB and BM.

Table 23: Average Agreement between Peripheral Blood and Bone Marrow – Overall *FLT3* Mutation Status

Measure of Agreement	Percent Agreement	95% CI[1]
APA	98.6%	(95.1%, 100.0%)
ANA	99.2%	(97.2%, 100.0%)

[1] The 95% CI was calculated using a non-parametric bootstrapping method (N=1000)

17.17.6. Peripheral blood and bone marrow agreement on *FLT3* mutation status is high, indicating that both specimen types are appropriate for use with the LeukoStrat CDx *FLT3* Mutation Assay. Detailed analyses of the agreement between peripheral blood and bone marrow for ITD and TKD can be found in the Summary of Safety and Performance (280544).

18. Clinical Performance Evaluation

18.1. IVS-056-001 Clinical Study (ADMIRAL Clinical Trial)

18.1.1. Study Overview (IVS-056-001)

18.1.1.1. The LeukoStrat CDx *FLT3* Mutation Assay has been developed by Invivoscribe (IVS) and is FDA approved as a companion diagnostic to be used as an aid in the assessment of Acute Myelogenous Leukemia (AML). In order to demonstrate clinical utility of the Companion Diagnostic (CDx) test, patients provided informed consent to have their sample tested with the LeukoStrat CDx *FLT3* Mutation Assay for enrollment in a pivotal clinical study (Phase III Study 2215-CL-0301 evaluating ASP2215 efficacy). The two types of mutations in the *FLT3* gene detected by the *FLT3* CDx test are internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations.

18.1.1.2. To assess the accuracy of the LeukoStrat CDx *FLT3* Mutation Assay, a next-generation sequencing method utilizing the MiSeq platform from Illumina served as an independent source of sequence information for the ITD and TKD mutations. The reference method testing was developed and validated by Invivoscribe for the ability to assess the presence or absence of *FLT3* ITD and TKD mutations. The assay was then used to assess the accuracy of the LeukoStrat CDx *FLT3* Mutation Assay using the extracted DNA of biological specimens collected during screening and enrollment of the 2215-CL-0301 study.

18.1.2. Study Objectives (IVS-056-001)

18.1.2.1. At the final analysis, the co-primary objective of the study was to estimate gilteritinib fumarate efficacy in the LeukoStrat CDx *FLT3* Mutation Assay positive population through application of a stratified log-rank test on overall survival.

18.1.2.2. The reference method study objective is to assess independently the presence or absence of *FLT3* mutations using the MiSeq next-generation sequencing platform from Illumina in order to confirm the accuracy of the LeukoStrat CDx *FLT3* Mutation Assay. The objective of this study is described in the Secondary Objective section of the protocol, Pivotal Study for the LeukoStrat *FLT3* Mutation Assay CDx for the ASP2215 Compound.

18.1.3. Patient Population (IVS-056-001)

18.1.3.1. At the final analysis, 771 specimens from 633 subjects were screened with the LeukoStrat CDx *FLT3* Mutation Assay. 371 subjects were included in the final Intent to Treat (ITT). Five subjects that tested negative by the LeukoStrat CDx *FLT3* Mutation Assay and enrolled based on local *FLT3* testing were excluded from the Full Analysis Set (FAS). Therefore, 366 subjects randomized to the study were used in the FAS for final analysis.

18.1.4. Selection of Samples for Reference Method Testing (IVS-056-001)

18.1.4.1. One sample per subject was selected for reference method testing. Samples with insufficient volume for the reference method test were excluded from the study. A total of 467 samples were tested by the reference method.

18.1.5. Safety Analysis (IVS-056-001)

18.1.5.1. 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.

18.1.6. Effectiveness (IVS-056-001)

18.1.6.1. At the final analysis, the median OS in the gilteritinib fumarate arm was longer (9.3 months) compared to that in the salvage chemotherapy arm (5.6 months) in the CDx+ population. The stratified hazard ratio (HR) by Cox regression was estimated to be 0.637 (95%CI 0.488, 0.830) relative to salvage chemotherapy, p-value (1-sided, stratified log rank) = 0.0004, corresponding to a relative risk reduction of death in favor of gilteritinib fumarate. The Kaplan-Meier plot is provided in Figure 8.
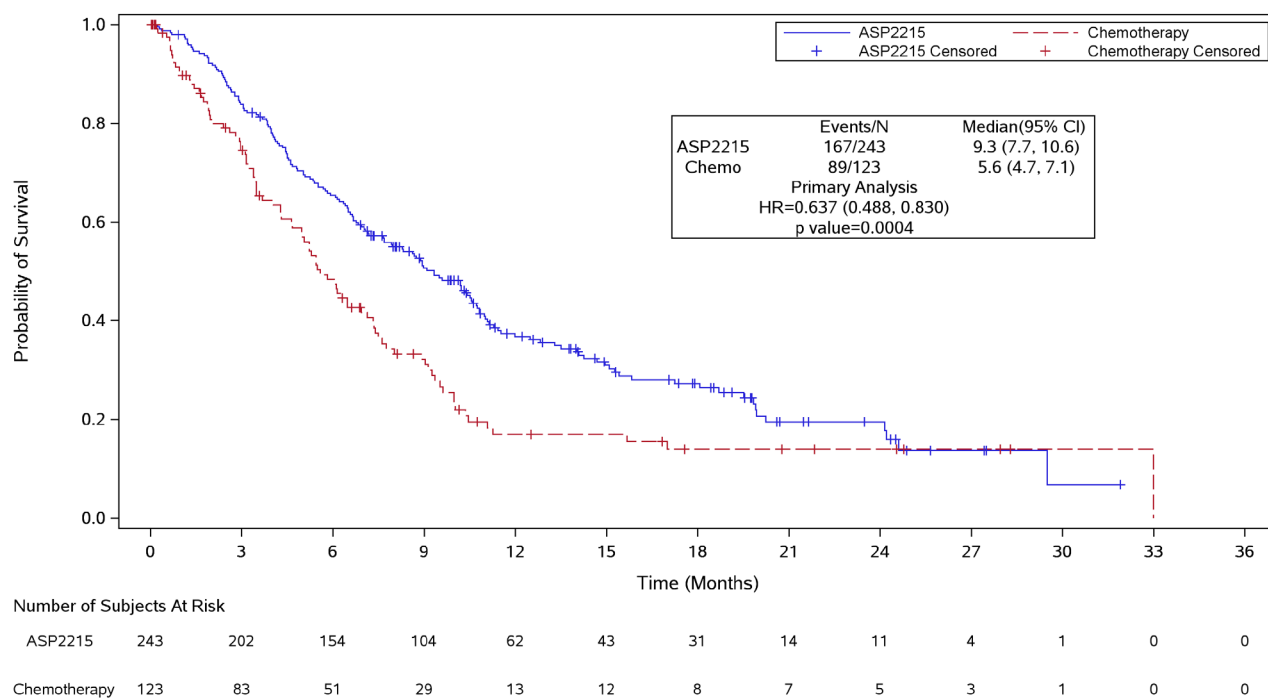
	Events/N	Median(95% CI)
ASP2215	167/243	9.3 (7.7, 10.6)
Chemo	89/123	5.6 (4.7, 7.1)

Primary Analysis
HR=0.637 (0.488, 0.830)
p value=0.0004

Number of Subjects At Risk

ASP2215	243	202	154	104	62	43	31	14	11	4	1	0	0
Chemotherapy	123	83	51	29	13	12	8	7	5	3	1	0	0

Figure 8 - Kaplan-Meier plot of overall survival.

18.1.6.2.   The LeukoStrat CDx *FLT3* Mutation Assay demonstrated agreement to the Reference Method. The overall agreement was high (97.2%). The lower bound of the OPA 95% confidence interval is above 90%, demonstrating agreement between the *FLT3* Mutation CDx and the MiSeq Sequencing assay.

Table 24: Agreement between CDx and MiSeq Sequencing

Agreement	Percent Agreement (N)	95% CI[1]
PPA	100% (300 / 300)	(98.8%, 100%)
NPA	92.0% (150 / 163)	(86.7%, 95.7%)
OPA	97.2% (450 / 463)	(95.2%, 98.5%)

[1]The 95% CI is calculated using Exact (Clopper Pearson) method.

The point estimates of PPA, NPA, and OPA for ITD are 100%, 92.8%, and 97% respectively. The point estimates of PPA, NPA, and OPA for TKD are 100%, 99.3%, and 99.4% respectively.

Table 25: Contingency Table between ITD CDx and MiSeq Sequencing

CDx	MiSeq		Total
	MiSeq+	MiSeq-	
CDx+	270	14	284
CDx-	0	180	180
Total	270	194	464

Table 26: Contingency Table between TKD CDx and MiSeq Sequencing

	MiSeq		Total
	MiSeq+	MiSeq-	
CDx+	32	3	35
CDx-	0	431	431
Total	32	434	466

18.1.6.3. Using the agreement data above (Table 24), the clinical performance of the device was determined as listed in Table 27.

Table 27: Clinical Performance

Measure of Clinical Performance	Calculated Performance
Diagnostic Sensitivity	$\frac{300}{300 + 0} = 1$
Diagnostic Specificity	$\frac{150}{150 + 13} = 0.92$
Positive Likelihood Ratio	$\frac{1}{1 - 0.92} = 12.5$
Negative Likelihood Ratio	$\frac{1 - 1}{0.92} = 0$

18.1.7. Conclusions (IVS-056-001)

18.1.7.1. At the final analysis, 366 subjects were included in the full analysis set. The median OS in the gilteritinib fumarate arm was longer (9.3 months) compared to that in the salvage chemotherapy arm (5.6 months) in the CDx+ population. The stratified hazard ratio (HR) by Cox regression estimated to be 0.637 (95%CI 0.488, 0.830) relative to salvage chemotherapy, p-value (1-sided, stratified log rank) = 0.0004, corresponding to a relative risk reduction of death in favor of gilteritinib fumarate.

18.1.7.2. For the reference method testing, the study acceptance criterion was met: The lower limit of the overall percent agreement (OPA) 95% two-sided exact (Clopper-Pearson) confidence interval exceeded 90%. The agreement between the LeukoStrat CDx *FLT3* Mutation Assay and the MiSeq next-generation sequencing reference method could be established.

18.2. IVS-062-005 Pivotal Bridging Study (QuANTUM-First AC220-A-U302 Clinical Trial)

18.2.1. Study Overview (IVS-062-005)

18.2.1.1. To support the safety and efficacy of the LeukoStrat CDx *FLT3* Mutation Assay (CDx), clinical agreement was required to be demonstrated between samples with *FLT3*-ITD status determined from the AC220-A-U302 Clinical Trial Assay (CTA) and the LeukoStrat CDx *FLT3* Mutation Assay in the intent to treat population. This pivotal bridging study corresponds to the Phase III AC220-A-U302 clinical trial of quizartinib hydrochloride in newly-diagnosed AML patients with *FLT3*-ITD mutations (QuANTUM-First). This bridging study demonstrates the device and drug safety and efficacy when AML patients are stratified with the LeukoStrat CDx *FLT3* Mutation Assay and treated with the quizartinib hydrochloride, which is likely to provide a benefit to subject outcomes without exposing the subjects to unreasonable risks. The LeukoStrat CDx *FLT3* Mutation Assay is intended to assist physicians in making treatment decisions for their AML patients with *FLT3*-ITD Mutations.

18.2.1.2. Description of Clinical Trial Assay

Navigate BP *FLT3*-ITD Mutation Assay (Navigate, Daiichi Sankyo Clinical Trial Assay). The Navigate BP *FLT3*-ITD Mutation Assay (Navigate, Daiichi Sankyo Clinical Trial Assay: CTA) was developed and validated by Navigate BioPharma Services Inc. (Carlsbad, CA). This is an in vitro diagnostic test intended for the qualitative detection of *FLT3*-internal tandem duplication (ITD) mutations from bone marrow or peripheral blood collected in K2 (EDTA) tubes using QIAmp DSP DNA Blood Mini Kit from Qiagen and subjected to polymerase chain reaction (PCR). *FLT3*-ITD mutations are identified by amplification of the region between exons 14 and 15 of the *FLT3* gene by PCR using a single set of DNA primers that flank the region. The PCR products are then subjected to capillary electrophoresis and fragment analysis on ABI 3130xl using GeneMapper software from ABI to resolve and detect the different-sized PCR products. Reaction products with ITD mutations that are greater in size than 330-base pair (bp) as well as non-mutant (ITD wild type) product of 330 bp are detected by the fragment analysis. The percent mutant (%Mutant) or variant allele frequency (VAF) for each sample is calculated by dividing the maximum mutant peak area under the curve (AUC) by the sum of the AUC of the mutant and non-mutant (330bp) peaks and multiplying by 100. A patient sample is considered positive if the VAF meets or exceeds the clinical cutoff of 3%. This assay was developed to only detect mutations caused by ITD insertions in the *FLT3* gene.

All testing is conducted in a CLIA laboratory following CLIA regulations and signed off by a pathologist for the *FLT3*-ITD Mutation Assay.

18.2.1.3. 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 quizartinib hydrochloride 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.

18.2.2. Study Objectives (IVS-062-005)

18.2.2.1. Demonstrate agreement with respect to selection of subjects that are *FLT3*-TD positive between the Clinical Trial Assay and LeukoStrat CDx *FLT3* Mutation Assay by assessing the positive and negative percent agreement (PPA and NPA, respectively) between the two assays.

18.2.2.2. Determine whether quizartinib prolongs overall survival (OS) compared to placebo in newly diagnosed AML subjects with *FLT3*-ITD mutation as determined by the LeukoStrat CDx *FLT3* Mutation Assay (CTA(+), CDx(+) population).

18.2.3. Patient Population (IVS-062-005)

18.2.3.1. 3468 patients were screened with the Daiichi Sankyo Clinical Trial Assay for enrollment into AC220-A-U302, with 1033 provided by the drug sponsor for the bridging study. Samples from 1029 subjects met the inclusion criteria for testing with the LeukoStrat CDx *FLT3* Mutation Assay. Of these 1029 subjects, 513 were CTA(+) and 516 were CTA(-). Once testing of the samples with the LeukoStrat CDx *FLT3* Mutation Assay was completed, 1023 subjects produced valid results in both the CTA and the CDx and six subjects produced invalid results with the CDx.

18.2.4. Safety Analysis (IVS-062-005)

18.2.4.1. 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.

18.2.5. Effectiveness (IVS-062-005)

18.2.5.1. The LeukoStrat CDx *FLT3* Mutation Assay demonstrated agreement to the CTA and comparable efficacy to the AC220-A-U302 study.

18.2.5.1.1. The primary analysis demonstrated:

Agreement between the LeukoStrat CDx *FLT3* Mutation and the CTA was demonstrated based on a subset of 1029 results. The point estimates for PPA and NPA were above 90%.

Table 28: Contingency between CDx and CTA

ITD CDx	ITD CTA		Total
	CTA(+)	CTA(-)	
CDx(+)	483	0	483
CDx(-)	27	513	540
Invalid	3	3	6
Total	513	516	1029

Invalid means that a sample was tested on the CDx assay but failed to return a valid result

Agreement with CDx invalids (95% CI) are:

- PPA 94.2% (91.8%, 96.0%)
- NPA 99.4% (98.3%, 99.9%)

18.2.5.1.2. Effectiveness in the (CTA(+), CDx(+)) population (483 subjects):

Quizartinib hydrochloride efficacy on overall survival (OS) in the CDx-positive population was evaluated. In the (CTA(+), CDx(+)) population, quizartinib treatment in combination with standard chemotherapy resulted in a clinically relevant improvement in OS compared to placebo. The median OS in the quizartinib arm was 29.4 months (19.1, NE) compared to 14.8 months (13.1, 26.2) for placebo resulting in 14.6 months prolongation of median OS (2-sided, stratified log rank p-value= 0.0640). The stratified hazard ratio (HR) was estimated to be 0.794 (0.621, 1.014) relative

to placebo corresponding to a 20.6% relative risk reduction of death in favor of quizartinib. The results were comparable to those observed in the QuANTUM-First (AC220-A-U302) study.

18.2.5.1.2.1. Effectiveness in the ITD Population (CTA(+)) population (539 subjects):

Quizartinib hydrochloride efficacy on overall survival (OS) in the CTA-positive population was evaluated. In the CTA(+), population, quizartinib treatment in combination with standard chemotherapy resulted in a clinically relevant improvement in OS compared to placebo. The median OS in the Quizartinib arm was 31.9 months (21.0, NE) compared to 15.1 months (13.2, 26.2) for placebo resulting in 16.8 months prolongation of median OS (2-sided, stratified log rank p-value= 0.0324). The stratified hazard ratio (HR) was estimated to be 0.776 (0.615, 0.979) relative to placebo corresponding to a 22.4% relative risk reduction of death in favor of quizartinib.

18.2.6. Conclusions (IVS-062-005)

18.2.6.1. Overall these results support that the LeukoStrat CDx *FLT3* Mutation Assay identifies the same AML patient population as enrolled in the AC220-A-U302 clinical trial with respect to *FLT3* ITD gene mutations.

19. Bibliography

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20. Technical and Customer Service

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21. Symbols

The following symbols are used in Invivoscribe diagnostic product labeling:

Symbol	Meaning	Symbol	Meaning
IVD	For In Vitro Diagnostic Use	CE	CE Mark
REF	Catalog Number	(hourglass)	Expiration Date
VOL	Reagent Volume	**EC REP**	Authorized Representative in the European Community
LOT	Lot Number	(factory)	Manufacturer
(storage)	Storage Conditions	(date)	Date of Manufacture
(consult)	Consult Instructions for Use	**TAQ**	Taq DNA Polymerase
CONTROL +	Positive Control	**EcoRV**	EcoRV Enzyme
CONTROL NTC	No Template Control	**NEBr3.1**	NEBuffer r3.1
CONTROL EC	Extraction Control	**MM**	Master Mix
REV	Revision	**UDI**	Unique Device Identifier
CH REP	Swiss Authorized Representative	**UKRP**	UK Responsible Person

22. Legal Notice

For Legal Notices related to this product, visit: https://invivoscribe.com/legal-notices/

23. Revision History

Table 29: IFU Revision History

Revision	Release Date	Change
A	February 2023	Initial Release
B	July 2023	Updated component NEBuffer 3.1 to r3.1 (new component) and included equivalency study. Updated error code IR07 to be reported as Invalid if generated a second time by the same sample. Update LeukoStrat Software to version 1.1.2.IVD and included its validation. The new software is designated as v1.1.x.IVD throughout this IFU. The third decimal place is the patch version and patches are minor problem fixes. Included how to change OS locale instructions for software v1.1.2.IVD. Removed EU AR address which is present on the physical packaging Minor formatting edits.
C	December 2023	Updated software system requirements applicable to version 1.1.3.IVD. Corrected DCS version in Table 2. Updated "LeukoStrat CDx *FLT3* Software" to full name in PFIGS IFUs. Removed how to change OS locale instructions for software v1.1.2.IVD.
D	August 2024	Addition of VANFLYTA® to intended use. Addition of Pivotal Bridging Study Overview to Clinical Performance Evaluation (IVS-062-005). Replacement of Legal Notice with link to Legal Notices webpage.
E	October 2024	Corrected DCS version in Table 2.
F	February 2025	Added symbols and definitions for additional regional representatives to Section 21.
G	March 2025	Removal of option to use Data Collection Software (DCS) for analysis. Included requirement to use GM v6.0 with patch B02.