LeukoStrat®

Instructions for Use

IVD

LeukoStrat® CDx *FLT3* Mutation Assay

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.

Rx Use Only

invivoscribe®

Catalog #	Products	Quantity
REF K4120361	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 is used as an aid in the assessment of patients with AML for whom RYDAPT® (midostaurin) treatment is being considered.

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

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

The test is for use on the 3500xL Dx Genetic Analyzer.

3. 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.

4. Summary and Explanation of the Test

Acute myelogenous leukemia (AML) in general has a poor prognosis. Many studies in AML have shown that the presence of *FLT3* activating mutations portends a poor prognosis 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 for use on the 3500xL Dx Genetic Analyzer with 3500 Dx Series Data Collection 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, TKD amplicon is enzymatically digested, and the amplicons are detected via capillary electrophoresis. *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 12: *Interpretation of Results*). A depiction of the workflow is shown in Figure 1.

5. Principles of the Procedure

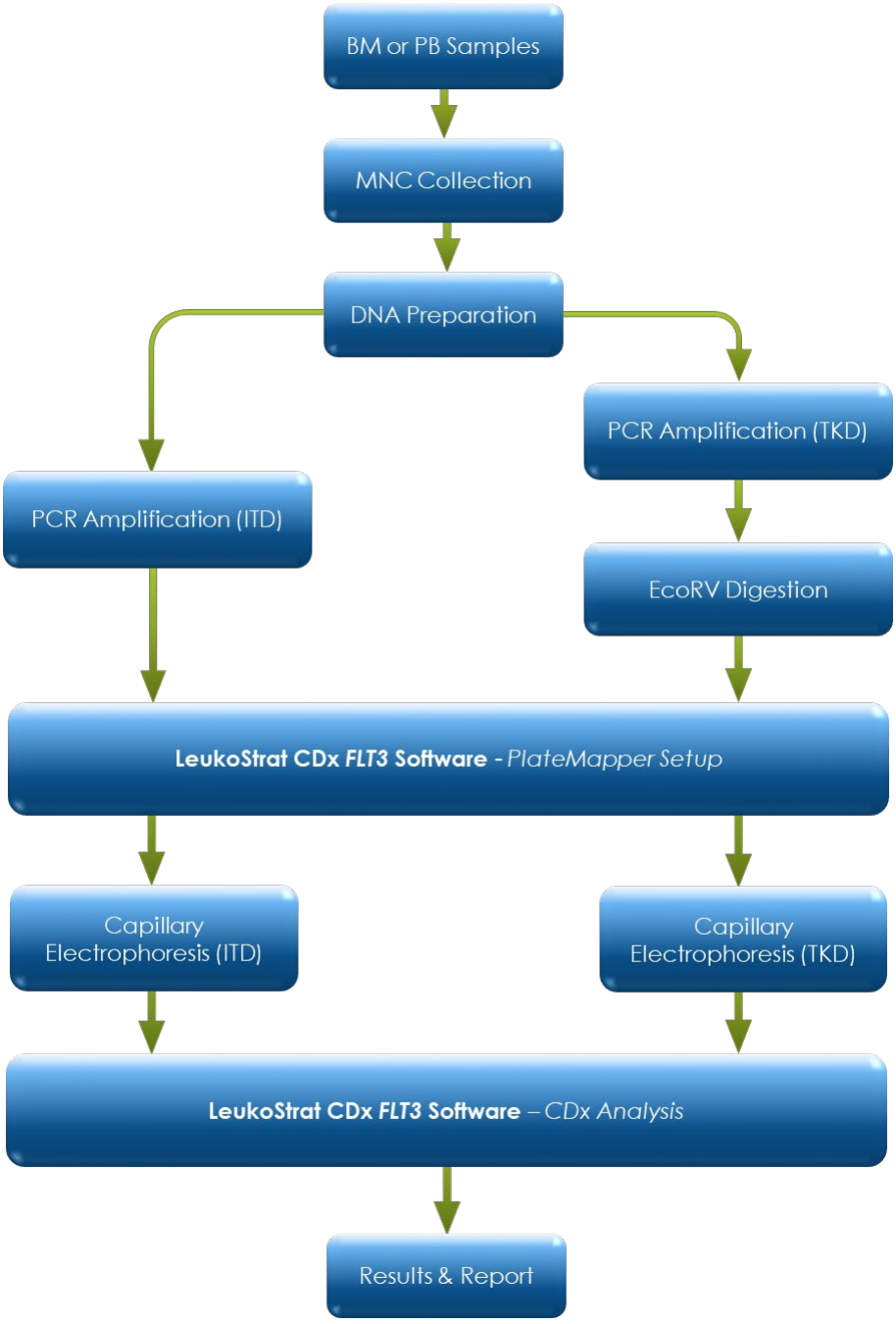


Figure 1: Workflow summary

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

FLT3 ITD or length mutations are caused by duplication and insertion of a portion of the *FLT3* gene that includes the region in and around the juxtamembrane (JM) region of the *FLT3* gene. These mutations vary in both the location and the length of the inserted duplicated DNA sequence. ITD mutations result in constitutive autophosphorylation and activation of *FLT3*.[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).

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

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

Wild-type alleles of the *FLT3* gene include an EcoRV restriction digest site. When a nucleic acid substitution occurs, the restriction digest recognition site disappears, and the EcoRV endonuclease is unable to identify and digest the DNA at this site. The LeukoStrat CDx *FLT3* Mutation Assay uses primers that lie on either side of the TKD region. The *FLT3* target region is amplified using PCR and then an EcoRV restriction digest is performed. One of the PCR primers is labeled with a fluorophore, 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).

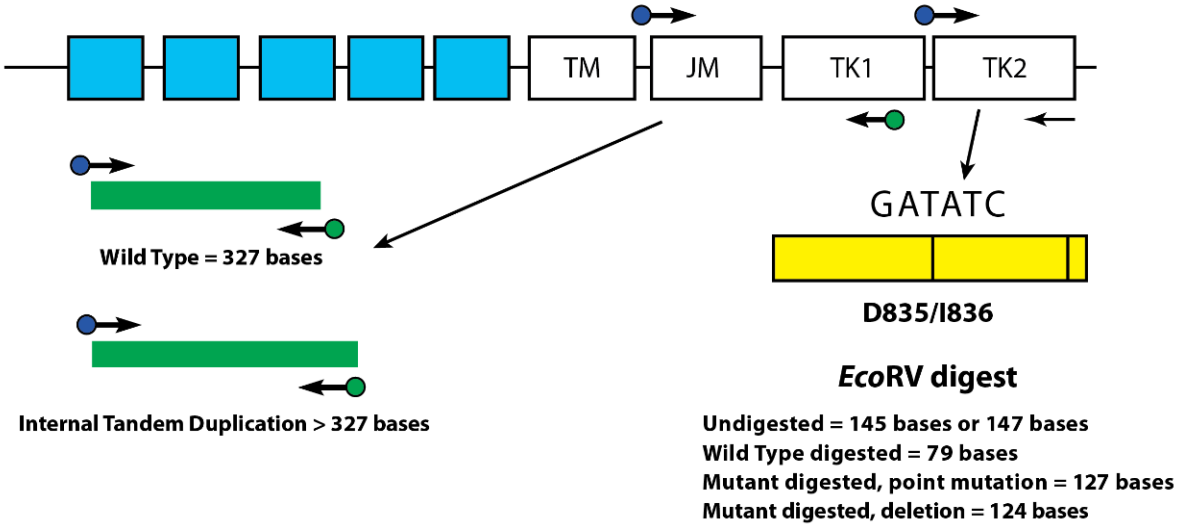
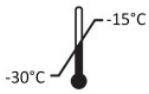


Figure 2: Depicted is a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target in and around the JM region for ITD or the activating loop of the kinase 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. 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 K4120361

Catalog Number	Reagent Name	Storage Temperature	Unit Quantity	# of Units / Kit
REF R0880080**	***FLT3* Extraction Control**		1800 µL/Vial	1 Vial
REF B4120051*	***FLT3* ITD Master Mix**		1500 µL/Vial	1 Vial
REF B4120061*	***FLT3* TKD Master Mix**		1500 µL/Vial	1 Vial
REF R0880060**	***FLT3* ITD Positive Control**		100 µL/Vial	1 Vial
REF R0880070**	***FLT3* TKD Positive Control**		100 µL/Vial	1 Vial
REF R0930020**	***FLT3* No Template Control**		200 µL/Vial	1 Vial
REF 261784	**Taq DNA Polymerase (Taq)**		200 µL/Vial	1 Vial
REF 261782	**EcoRV Enzyme**		200 µL/Vial	1 Vial
REF 261984*	**NEBuffer r3.1**		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)

Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
Hi-Di Formamide	Thermo Fisher Scientific: • Hi-Di™ Formamide 3500 Dx Series	4404307	N/A
LIZ Size Standard	Thermo Fisher Scientific: • GeneScan™ 600 LIZ® dye Size Standard v2.0 - Dx	A25794	N/A
Polymer	Thermo Fisher Scientific: • POP-7™ Polymer (384 Samples) 3500 Dx Series	4393709	N/A
Buffer	Thermo Fisher Scientific: • Anode Buffer Container 3500 Dx Series Thermo Fisher Scientific: • Cathode Buffer Container 3500 Dx Series	4393925 4408258	N/A
Capillary Electrophoresis Instrument and Software	Thermo Fisher Scientific: • 3500xL Dx Genetic Analyzer with 3500 Dx Series Data Collection Software (DCS) 3 IVD v3.2 • Patch "3500 Dx Series Data Collection Software Patch v1.0 - Peak Height" must be applied to DCS	A46345	Dx Fragment Analysis Module Software Required. Patch can be requested from Thermo Fisher Scientific
Capillary Array	Thermo Fisher Scientific: • 3500xL Dx Capillary Array 50 cm	4404688	N/A
Septa	Thermo Fisher Scientific: • 3500 Dx Series Septa Cathode Buffer Container Thermo Fisher Scientific: • 3500 Dx Series Septa 96-well	4410716 4410700	N/A
Retainer & Base Set for 3500xL Dx	Thermo Fisher Scientific: • 3500 Dx Series 96-well Standard Retainer & Base Set	4410227	N/A
Spectral Calibration Dye Set	Thermo Fisher Scientific: • DS-33 Matrix Standard Set (Dye Set G5) - Dx	A25775	N/A
Calibrated Pipettes	• Single Channel 5 - 120µL • 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	4452300 (VRTI DX 200-Q)	N/A

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

Reagent/Material	Reagents/Materials and Suppliers	Catalog #	Notes
Vortex Mixer	N/A	N/A	N/A
PCR Plates or Tubes	N/A	N/A	Sterile, Skirted Plates
Filter Barrier Pipette Tips	N/A	N/A	Sterile, 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	Cell Counting System	N/A	Capable of measuring 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
DNA Extractor System	QIAgen: • QIAcube System (110 V)	9001882	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
3mL Vacutainer Collection Tube (no additive)
Disposable transfer pipettes
Pipette tips
96-Well Capillary Electrophoresis Plates, Skirted

7. Instruments/Accessories

NOTE: The assay is for use on the 3500xL Dx Genetic Analyzer with 3500 Dx Series Data Collection Software
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

7.1. Software (Provided)

7.1.1. REF : K4120371 – LeukoStrat CDx *FLT3* Software v1.1.x.IVD

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.

7.1.1.1. Computer Requirements:

- Operating System: Windows™ 10 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, 2023

7.1.2. REF : K4120401 – Invivoscribe LeukoStrat CDx Assay Installer v1.0

8. Warnings and Precautions

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

- IVD This product is for In Vitro Diagnostic Use.
- For prescription use only.
- The assay is only validated for use on the 3500xL Dx Genetic Analyzer with 3500 Dx Series Data Collection Software. 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 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 3500Dx instrument if crystallization is observed, as crystallization may impact the performance of the LeukoStrat CDx *FLT3* Mutation Assay and 3500Dx instrument. 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.
    - A false negative assay result could cause an AML patient not to experience any potential benefit that might be associated with receiving XOSPATA® (gilteritinib), RYDAPT® (midostaurin), or VANFLYTA® (quizartinib) treatment. However, the patient would receive intense chemotherapy as the standard therapy for AML.
    - Patients with a false positive assay result may receive treatment with XOSPATA® (gilteritinib), RYDAPT® (midostaurin), or VANFLYTA® (quizartinib) 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.

8.1. Cyber Security Precautions

- 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 the local user to prevent unauthorized use of the software.
- Ensure Windows and Adobe Acrobat Reader are always updated to the latest available security patches.
- 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:
  - Symantec Endpoint Protection Version 14.3
  - McAfee Endpoint Security Version 10.7
  - ESET Endpoint Security Version 10.0

9. Specimen Collection and Preparation

9.1. 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.

9.2. PCR-Interfering Substances

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

9.3. 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).

10. Assay Procedure

10.1. Specimen Inspection

10.1.1. Unpack peripheral blood (PB) and/or bone marrow aspirate (BM) specimens and reject any specimens that do not meet the requirements listed in section 9.3

10.2. Sample Processing Preparation

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

10.2.2. 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.

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

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

10.2.3.1. 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.

10.2.4. 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.

10.3. 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.

10.3.1. 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.

10.3.2. 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.

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

10.4. Mononuclear Cell (MNC) Isolation

10.4.1. 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.

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

10.4.3. 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

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

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

10.4.6. 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.

10.4.7. Centrifuge the conical tubes under the following conditions:

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

10.4.8. 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.

10.4.9. 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.

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

10.5. Mononuclear Cell Count

10.5.1. Use a suitable mononuclear cell counting system to determine the concentration of mononuclear cells per sample. Ensure installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions.

10.6. Preparing Samples for DNA Extraction and Isolation Completion

10.6.1. If the reported concentration is ≤ 5 million cells/mL, the entire cell suspension volume is processed. Proceed to step 10.6.3.

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

10.6.2.1. 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)
- $V_i = \frac{5{,}000{,}000\frac{cells}{mL} x\ 1\ mL}{C_i}$

10.6.2.2. 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.

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

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

10.6.3. 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

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

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

10.6.6. 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.

10.7. 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.

10.7.1. 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.

10.7.1.1. 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.

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

10.7.2.1. 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.

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

10.7.3. 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.

10.7.4. 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.

10.8. 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.

10.8.1. Pipette the cell suspensions (from step 10.6.6) 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.
10.8.2. Place the Extraction Control sample tube in the last position of the run.
10.8.3. Load all of the remaining sample tubes, the reagents, and the aliquoted Protease solution into the instrument.
10.8.4. Start the run, ensuring the following selections are made.

10.8.4.1. Use the QIAamp DNA Blood Mini protocol.
10.8.4.2. Select **Blood** or **body fluid** as the starting material.
10.8.4.3. Set the *Elution volume* to **100 µL**.

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

10.9. Quantification and Dilution of DNA

10.9.1. 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.
10.9.2. 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.
10.9.3. Blank the instrument using 2 µL of AE buffer.
10.9.4. Read 2 µL of each DNA sample in singlicate.
10.9.5. 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.

10.9.6. 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 5 freeze thaw cycles.

10.9.7. 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 *(Ci)*	Final Volume *(Vf)*
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

10.10. 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.

10.10.1. 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.

10.10.2. 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.

10.10.3. 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 2.

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

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

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

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

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

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

10.10.8. 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.

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

10.10.10. 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.

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

10.10.12. 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%.		

10.10.13. 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.

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

10.10.15. 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 10.11. *Restriction Digest (TKD Mutation Only)* and for ITD plates, continue to section 10.12. *Capillary Electrophoresis Detection*.

10.11. 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.

10.11.1. Allow a NEBuffer r3.1 tube to thaw at room temperature.
10.11.2. While reagents are warming to room temperature, label a 96-well plate with TKD digestion and a unique identifier.
10.11.3. 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 4.

10.11.3.1. 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 6 samples added to Y allow for pipetting error.

10.11.4. Vortex the NEBuffer r3.1 tube at MAX for 5 to 15 seconds.
10.11.5. Remove the EcoRV from the -30°C to -15°C storage. Do not vortex.
10.11.6. Using a microcentrifuge, centrifuge all tubes (including EcoRV) for 2 to 5 seconds to remove liquid from the lids.
10.11.7. Add the calculated volumes of NEBuffer r3.1 and EcoRV to a labeled tube of appropriate volume.
10.11.8. Mix the solution by pipetting up and down 5 to 10 times. Place EcoRV back in storage at -30°C to -15°C.
10.11.9. Aliquot 1.5 µL of the digestion mix solution to the appropriate wells of the digestion plate.
10.11.10. 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.
10.11.11. 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.
10.11.12. Centrifuge the plate at 1400×g for 1 minute.
10.11.13. Place the digestion plate in a Veriti Dx thermal cycler and close the lid.
10.11.14. 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 ∞

10.11.15. 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.
10.11.16. 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 10.12).

10.12. 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 Dx runs in sets of 24 capillaries, called an injection, which comprises 3 columns by 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.

10.12.1. All 3500xL Dx steps including installation, operation, calibration, cleaning and maintenance procedures are performed according to the manufacturer's instructions unless stated otherwise below.
10.12.2. ITD and TKD assays must be run on different injections and with different injection conditions. The 3500xL Dx ITD and TKD conditions are listed in Table 6. These settings are saved in the 3500xL Dx for future use.

Table 6: 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	

Table 6: 3500xL Dx Genetic Analyzer Conditions

Parameter	ITD CDx Assay Parameters	TKD CDx Assay Parameters
Run Time	1630 sec.	
Run Voltage	19.5 kVolts	
PreRun Time	180 sec.	
PreRun Voltage	15 kVolts	
Data Delay	1 sec.	

10.12.3. Click **Refresh** to update the consumables' time on the instrument and number of injections performed in the 3500xL Dx Dashboard. Check the 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 Dx Materials

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

10.13. Prepare Size Standard Solution, if needed

10.13.1. The size standard solution consists of a mixture of LIZ Size Standard and Hi-Di Formamide.
10.13.2. Remove a tube of size standard solution from 2°C to 8°C storage, if available, and proceed to step 10.13.6. If not, make a size standard solution tube by performing the next three steps.
10.13.3. Allow a bottle of Hi-Di Formamide to thaw at room temperature. Remove a tube of LIZ Size Standard from storage.
10.13.4. Vortex the tubes at MAX for 5 to 15 seconds. Centrifuge the tubes for 2 to 5 seconds in a microcentrifuge.
10.13.5. Add 56 µL of LIZ Size Standard to 1 mL of Hi-Di Formamide. Label the size standard solution tube with the date.
10.13.6. 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.

10.14. Prepare Sample Plate

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

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

10.14.2. Label a 96-well plate with ITD CE and/or TKD CE, as applicable, and a unique identifier.
10.14.3. Determine the number of wells needed for a run.

- Number of wells $= 24X$
  - $X =$ the number of injections.
- Calculate the volume of size standard solution needed.
  - Maximum volume of size standard solution $= 9.5\ \mu L \times (24X + 4)$
  - The additional 4 samples added to X allow for pipetting error.

10.14.4. 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.

10.14.5. 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.

10.14.6. Seal the CE plate with a foil seal and centrifuge the plate at 1400×g for 1 minute.
10.14.7. Place the CE plate in a Veriti Dx thermal cycler and close the lid.
10.14.8. Program the thermal cycler with the steps listed below (ramp rate 75%).

- Step 1: 95°C for 3 minutes

10.14.9. Step 2: 4°C for 5 minutes
10.14.10. 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.
10.14.11. 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.
10.14.12. Prepare each plate assembly by placing the CE plate on a 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 Dx 96-well plate retainer.

10.15. Set up PlateMapper with LeukoStrat CDx *FLT3* Software

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

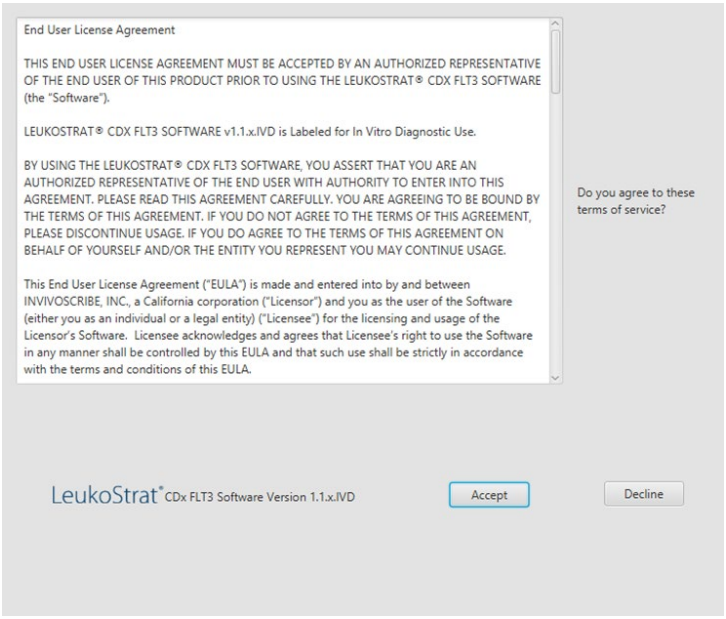
10.15.1. Install the LeukoStrat CDx *FLT3* Software.

10.15.1.1. Copy *LeukoStratCDx-1.1.x.IVD.msi* installer from the software CD to a local drive on your computer.
10.15.1.2. Double-click the **LeukoStratCDx-1.1.x.IVD.msi** file.

10.15.1.2.1. If a *Microsoft Defender SmartScreen* message appears after double-clicking the msi file, Click **More info**.
10.15.1.2.2. Verify the publisher is Invivoscribe, Inc. To proceed with installation, click **Run anyway**.

10.15.1.3. The *LeukoStratCDx-1.1.x.IVD.msi* setup wizard box will appear. Click **Next**.
10.15.1.4. The default installation location is *C:\Invivoscribe\LeukoStratCDx-1.1.x.IVD*. Click **Next**.
10.15.1.5. Click **Install**. The installation will begin.
10.15.1.6. A *User Account Control* dialog box will appear. Click **Yes**.
10.15.1.7. Click **Finish** to exit the setup wizard.

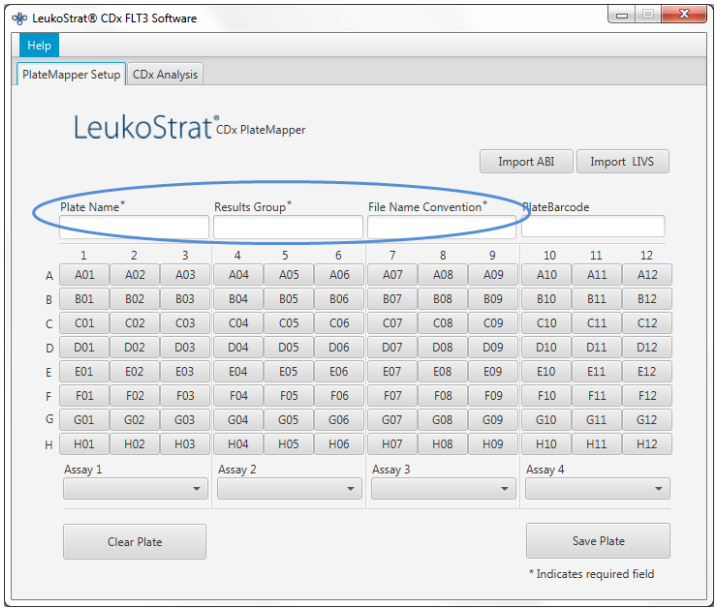
10.15.2. Open the *LeukoStrat CDx FLT3 Software*. Click **Accept** (Accept) to agree to terms of service.



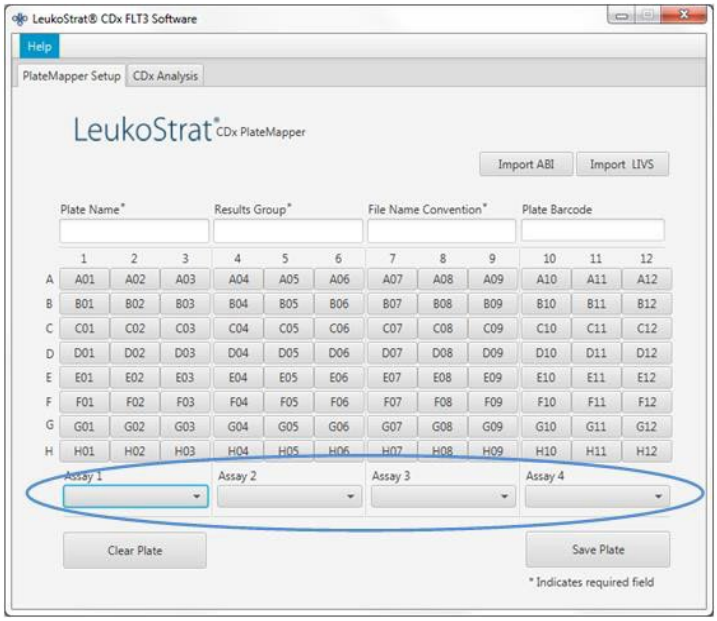
10.15.3. Within the PlateMapper Setup, fill in the three required fields located above the plate map. These required fields are Plate Name, Results Group, and File Name Convention (circled below).

10.15.3.1. *Plate* map names may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.

10.15.3.2. *Results Group* and *File Name Convention* entries must match the names of corresponding user-programmed entries on the 3500xL Dx (selected in step 10.16.14).

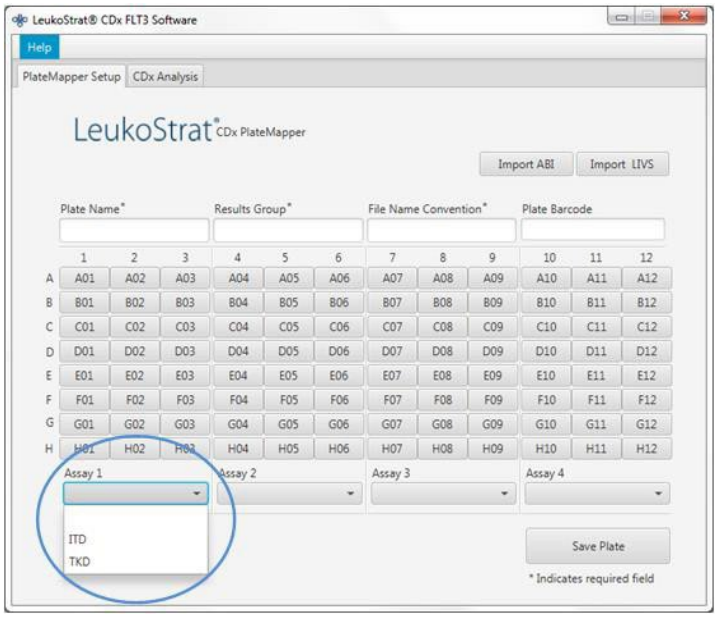


10.15.4. The plate map has four assays allowed per plate (3 columns per assay). Each assay corresponds with the injection that will occur during the 3500xL Dx run. Only one assay may be run per injection (ITD or TKD).



10.15.5. Select the assay from the drop down menu (

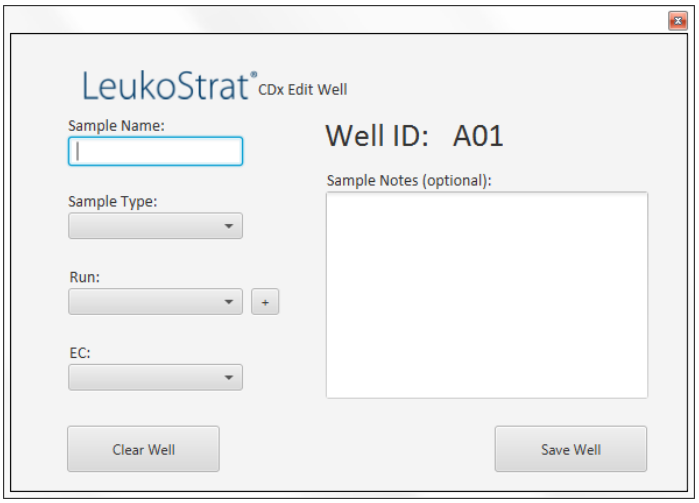
) which corresponds with the samples that are located above it on the *PlateMapper Setup* screen.



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

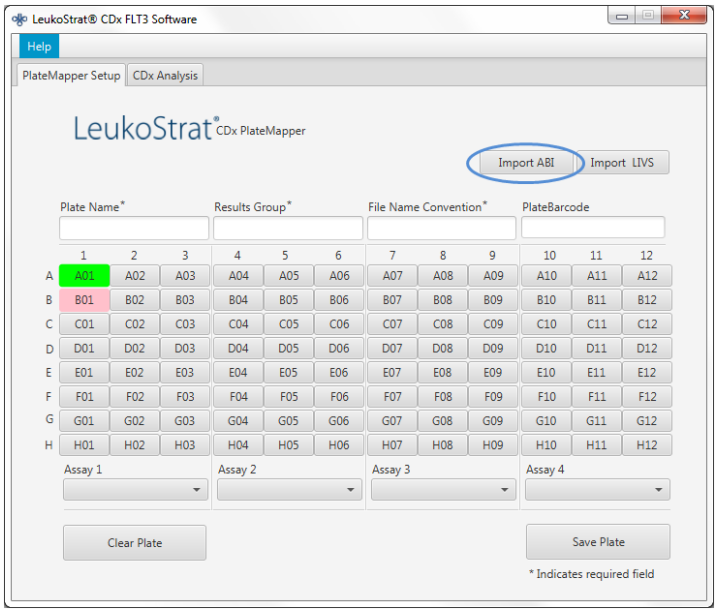
NOTE: When entering well information, enter the *Extraction Control (EC), Positive Control (PC),* and *No Template Control (NTC)* first. Controls may be placed anywhere on the plate, not necessarily in the first three wells. Enter the information for the SAMPLE wells afterwards, as they will require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

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



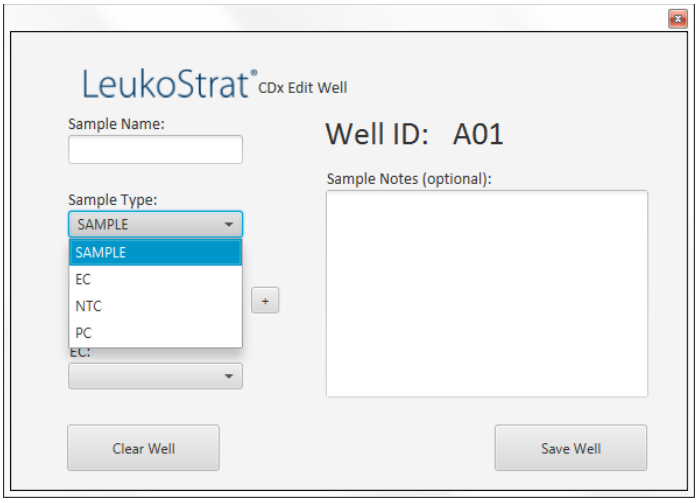
10.15.7. Enter a *Sample Name* that describes the well. Sample names may only contain 50 characters or less, made up of [A-Z, a-z, 0-9], single spaces, and hyphens.

10.15.7.1. 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.



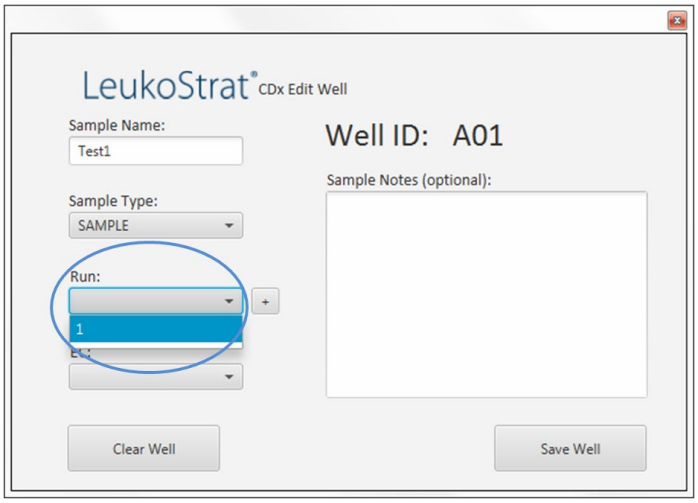
10.15.8. Select the **Sample Type** for each well from the drop down menu. The options to select are:

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

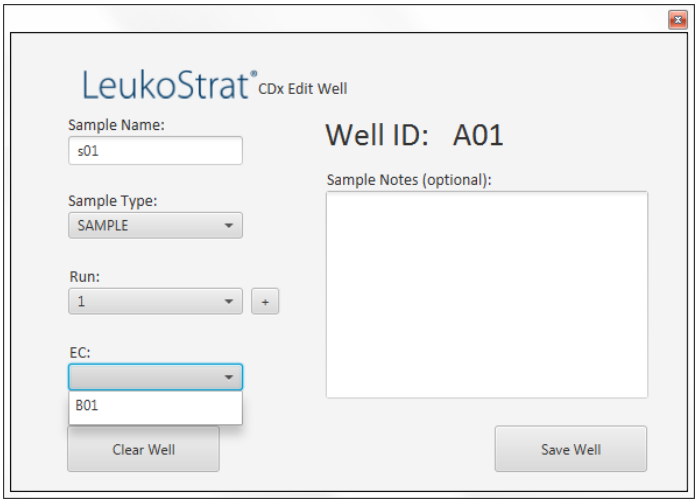


10.15.9. Select the **Run Number** from the drop down menu. To add a new run number, click the "+" sign next to the drop down menu.

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.

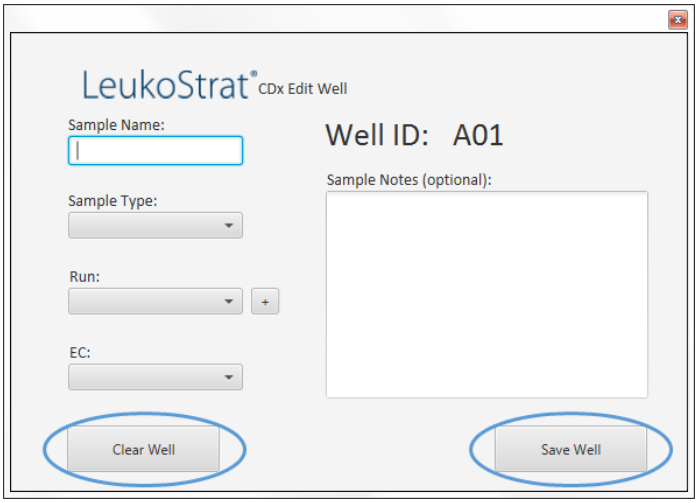


10.15.9.1. Select the associated **EC** from the drop down menu (only required if the *Sample Type* is *SAMPLE*). Up to 11 samples may be associated with a single Extraction Control.



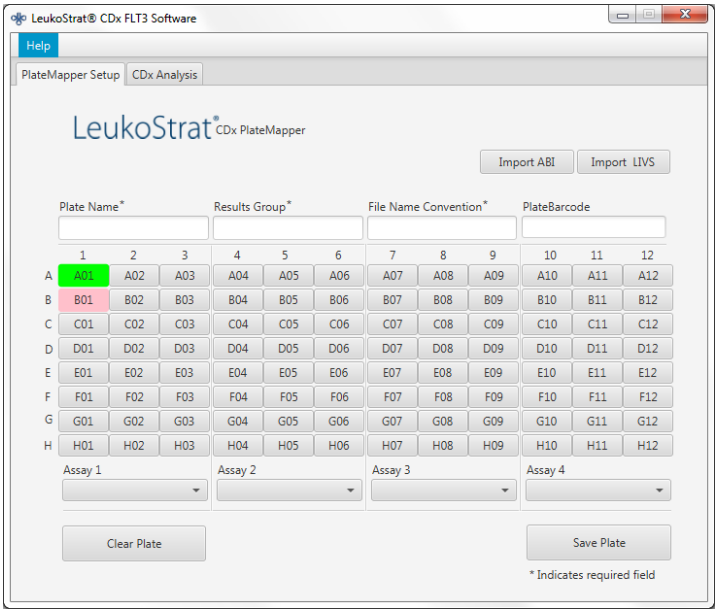
10.15.10. Additional comments about the sample or control may be entered in the *Sample Notes* field. These comments will appear in the *Sample Report*.

10.15.11. 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**.



10.15.12. 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.



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

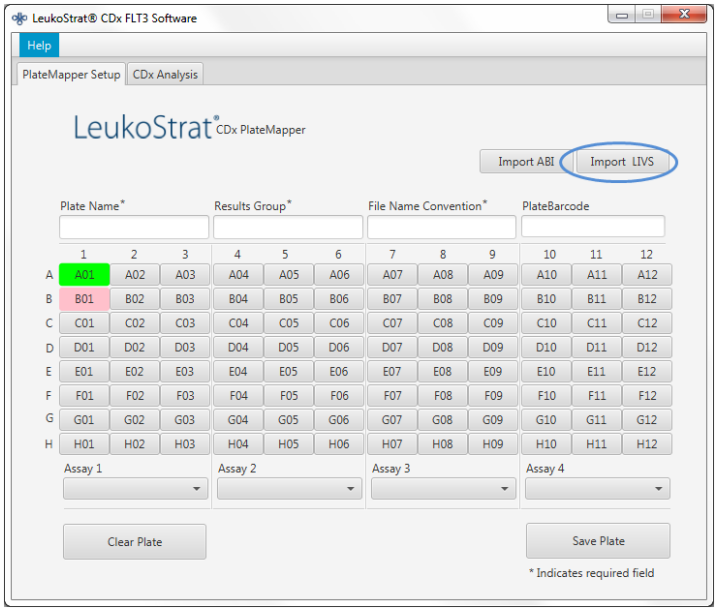
10.15.14. Once all wells have been entered, click **Save Plate,** and the user will be prompted for a location to save the ABI file (3500 Plate Layout File Version 1.0) and LIVS file generated by the software. One ABI file and one LIVS file will be generated for every plate setup.

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 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.

10.15.14.1. 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.



10.15.15. The user will continue with the software after the 3500xL Dx run has completed.

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

10.15.17. 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 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 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 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.

10.16. Set Up 3500xL Dx Software

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

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

10.16.2. If the Assays Library does not already contain *ITD CDx Assay* and *TKD CDx Assay*, run the Invivoscribe LeukoStrat CDx Assay Installer.

NOTE: The installer will overwrite any assays with the same name.

10.16.2.1. Exit the Data Collection Software user interface and stop the web services (right click the green check mark in the system tray and select **Exit**).

10.16.2.2. Insert the memory stick containing the Invivoscribe LeukoStrat CDx Assay Installer into the computer running the Data Collection Software. Navigate to the folder containing the installer file and double click the file to begin installation. Proceed through the installation prompts until a popup appears with the following restart instructions:

10.16.2.2.1. Power off the 3500xL Dx instrument.
10.16.2.2.2. Restart the computer. After the Windows operating system log on screen appears, power on the 3500xL Dx instrument.
10.16.2.2.3. Wait for the 3500xL Dx status light to turn solid green, then log on to the Windows operating system.
10.16.2.2.4. Start the *3500 Dx Data Collection* software after the 3500 Dx services have started.
10.16.2.2.5. Perform a *Spatial Calibration* run to verify proper operation of the 3500xL Dx instrument.

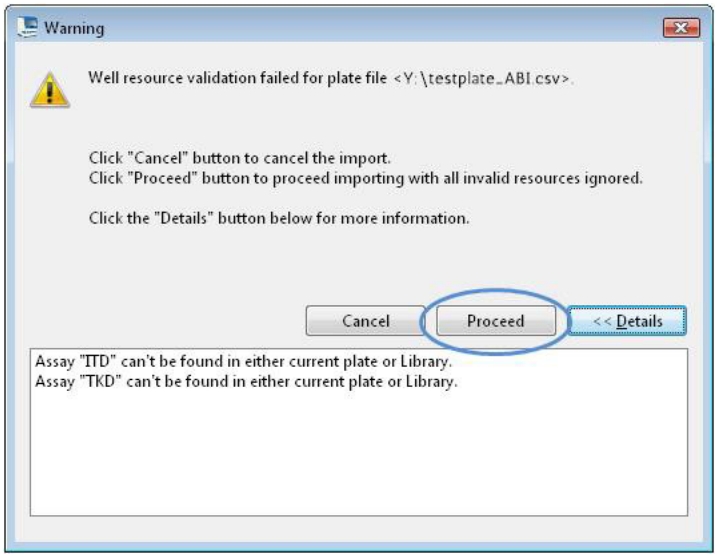
10.16.2.3. Perform the restart instructions as directed. The following items should now be available within the Data Collection Software libraries.

- Contact Invivoscribe Technical Support if installer did not perform as expected.

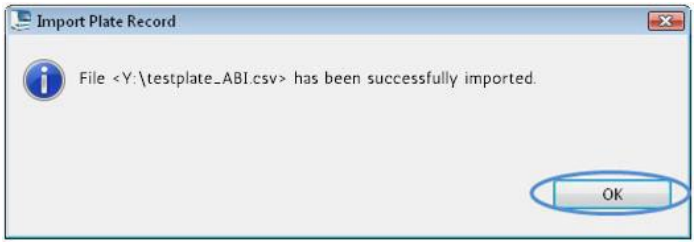
10.16.2.3.1. Assays Library: ITD CDx Assay and TKD CDx Assay
10.16.2.3.2. File Name Convention Library: *FLT3 CDx FNC*
10.16.2.3.3. Results Group Library: *FLT3 CDx RG*

10.16.3. From the 3500xL Dx dashboard screen, click on the **Create New Plate** button.
10.16.4. For the *Plate Name*, enter a short descriptor.
10.16.5. Ensure the number of wells is set to *96*.
10.16.6. For the plate type, select **Fragment** from the drop down menu.
10.16.7. Ensure the capillary length is *50 cm*, and the polymer is *POP7*.
10.16.8. Enter the operator initials in the *Owner* section.
10.16.9. Click Assign Plate Contents.
10.16.10. 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.

10.16.10.1. If there is no match in the 3500xL Dx library for the assay name in the ABI file, click **Proceed** in the popup that appears:



10.16.11. Click **OK** in the following popup window.



10.16.12. 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 Dx.

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

10.16.13. Confirm the *Assays* programmed in the 3500xL Dx use the parameters listed in Figure 3 for the **ITD CDx Assay** or **TKD CDx Assay**.

- Ensure that within the *Sizecalling Protocol* settings, *GS600LIZ* is set as the *Size Standard* and the other settings match those given in Figure 3.

10.16.14. 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.

10.16.15. Load the plate(s) on the 3500xL Dx.
10.16.16. Click **Link Plate for Run**. Operator may save changes to plate if prompted. If a second plate is to be run, repeat steps 10.16.2 through 10.16.15.

10.17. Run 3500xL Dx Genetic Analyzer

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

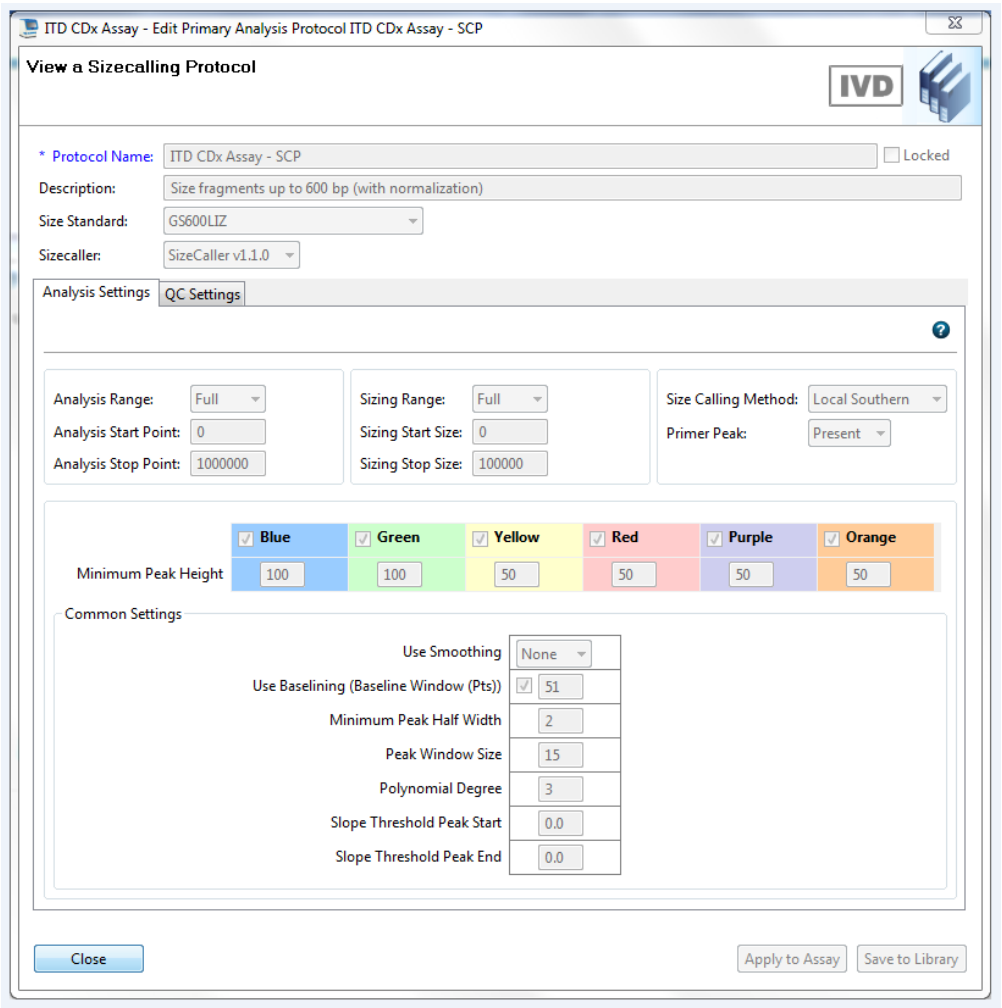


Figure 3: Size calling Protocol settings for ITD. For TKD, settings are identical except that *Polynomial Degree* is set to **5**

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

10.18. Export Data from ABI3500xL Dx Data Collection Software (DCS)

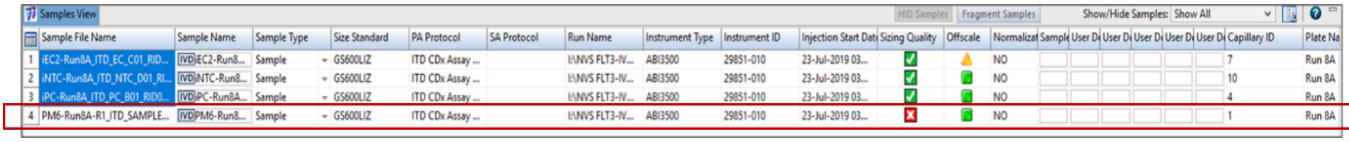
NOTE: It is a requirement to use DCS with the patch "3500 Dx Series Data Collection Software Patch v1.0 - Peak Height." The patch should be applied on top of DCS. Contact the manufacturer to receive the patch and installation instructions.

10.18.1. Ensure patch "3500 Dx Series Data Collection Software Patch v1.0 - Peak Height" has been applied.

10.18.1.1. To confirm patch has been applied, open DCS. Navigate to the *Help* tab and select **About 3500 Dx Series Data Collection Software 3 IVD v3.2**. In the **About** pop-up, the *SizeCaller* version will read v1.1.0.1 if the patch is installed.

10.18.2. In DCS, under the *Workflow* tab, click **View Fragment/HID Results**.

10.18.3. Ensure the desired samples to be analyzed are selected in the top window. Samples may be added by selecting **Import** and browsing to the FSA files for the run. **If any samples show a failure (X) in the *Sizing Quality* column, they must be deselected before export.**



NOTE: Failure to deselect samples with a failure (X) in the *Sizing Quality* column may result in incorrect sample results.

10.18.4. Ensure dye colors *Red, Green,* and *Blue* are checked in the *Plot View* window. Ensure dye colors *Orange* and *Yellow* are not checked in the *Plot View* window.

10.18.5. Use the dropdown menu in the *Sizing Table View* to select **Show Selected Dye Peaks**.

10.18.6. Ensure that in the *Sizing Table View* window that the only columns showing are *Dye/Sample Peak*, *Sample File Name*, *Size*, *Height*, and *Area in Point*. If not, click the button in the top left corner of the sizing table and remove or add the necessary columns. Adjust the order of the columns to match the order provided in Figure 4.

Sizing Table View | Annotation View

	Dye/Sample Peak	Sample File Name	Size	Height	Area in Point
1	B, 1	Sample10_InstallFragmentPlate_B...		322	2616
2	B, 2	Sample10_InstallFragmentPlate_B...	9.09	24421	122843
3	B, 3	Sample10_InstallFragmentPlate_B...	10.52	726	4093
4	B, 4	Sample10_InstallFragmentPlate_B...	14.94	21269	102309

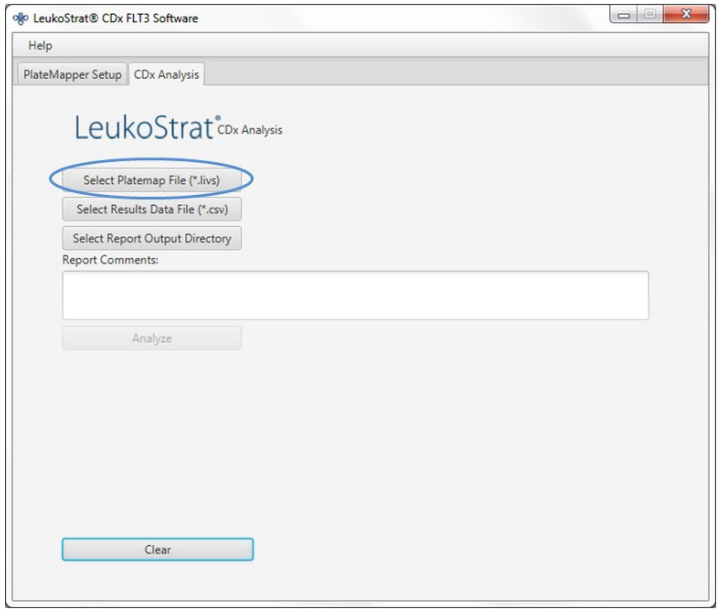
Figure 4. Example Sizing Table

10.18.7. Click **Export Results** and save export as a CSV file.

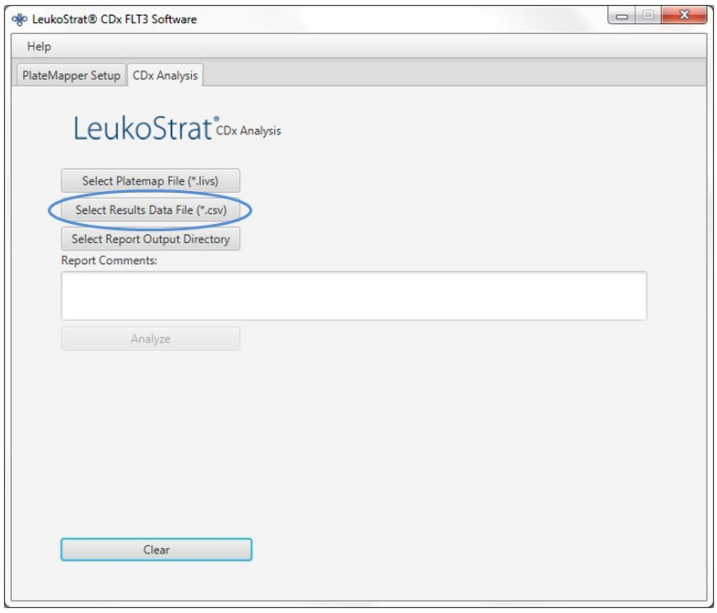
NOTE: Do not edit the CSV file in any way.

10.19. Data Analysis with LeukoStrat CDx *FLT3* Software

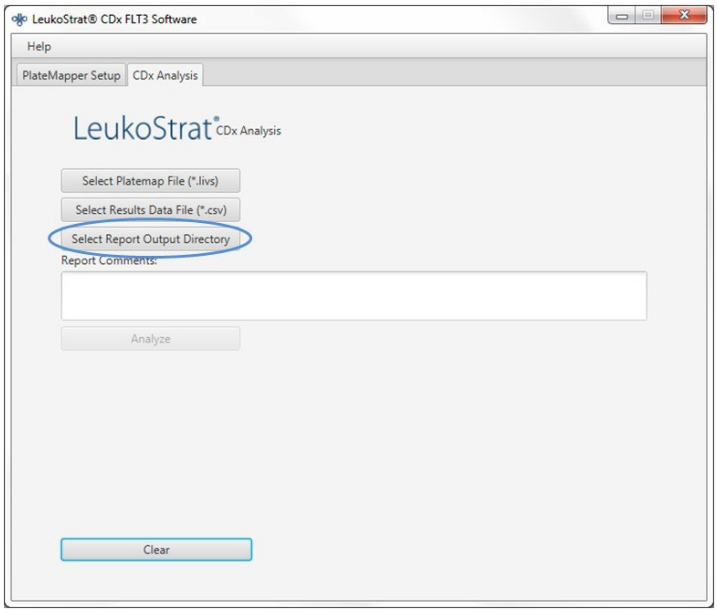
10.19.1. Open the LeukoStrat CDx *FLT3* Software, accept the licensing agreement, and click on the *CDx Analysis* tab of the LeukoStrat CDx *FLT3* Software. Click **Select Platemap File (*.livs)** and navigate to the LIVS file generated from the *PlateMapper Setup* tab.



10.19.2. Click the **Select Results Data File (*.csv)** and select an exported CSV file from step 10.18.7 for analysis.



10.19.3. Click **Select Results Output Directory** and choose a destination folder for the results.

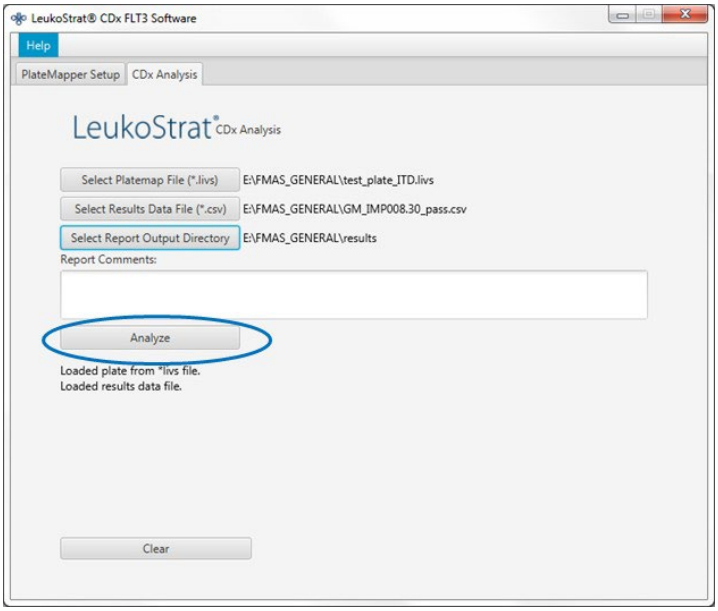


10.19.3.1. Additional comments about the run, samples or controls can be entered in the *Report Comments* field. These comments will appear in the *Run Report*.

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.

10.19.4. 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 5, Figure 6, and Figure 7).

- 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

Figure 5. 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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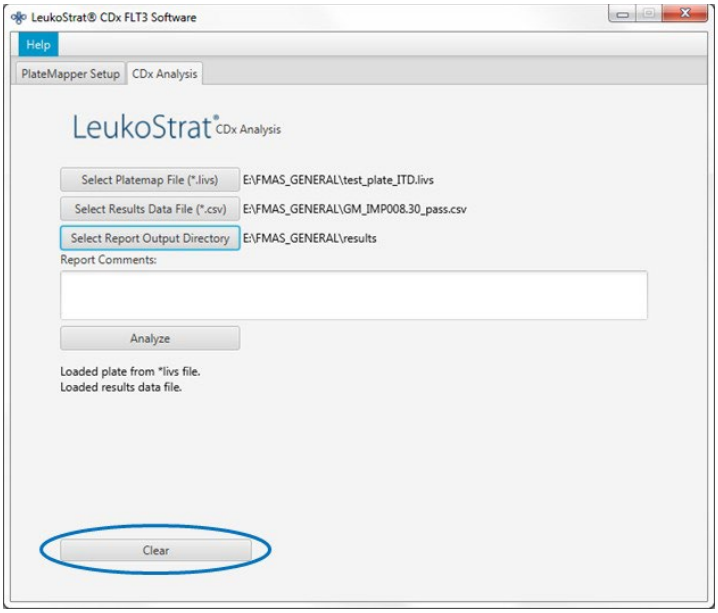
Figure 6. Example *Sample Report.*

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-bb66-72c79c6c5c13	SAMPLE	d2a45feb-9d24-42c8-b2d0-4e614e4d9b70	SampleA11_ITD_SAMPLE_A11	FAIL	N/A		v1.1.x.IVD

Figure 7. Example *CSV run export file*

10.19.5. Click **Clear** to reset all fields.



10.19.6. 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 Data Collection Software Analysis step.	Ensure that only Red, Green, and Blue dyes are selected during the Data Collection 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 Data Collection Software analysis step	Make sure to select red dye during Data Collection Software analysis step.
• Unrecognized data results file format. [AD03]	Data Collection Software file is corrupted	Do not edit the Data Collection 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.
• 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 10.16.14 this is done).	Repeat run beginning with step 10.12: *Capillary Electrophoresis Detection* with correct File Name Convention
• String index out of range: 15	CSV results file have been edited.	Repeat export of CSV results file from DCS. Do not edit the CSV file in any way.

11. Quality Control

11.1. Run Validity

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

11.1.2. 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 13: *Retesting*).

11.2. Extraction Control and Sample Validity

11.2.1. 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*.

11.2.2. 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 13: *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 13: *Retesting*).

12. Interpretation of Results

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

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

12.3. 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.

12.4. 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.

12.5. For determination of overall *FLT3* mutation status or whether midostaurin or gilteritinib are indicated:

12.5.1. 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 midostaurin or gilteritinib is indicated.

12.5.2. 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 midostaurin or gilteritinib is not indicated.

12.5.3. 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

12.6. For determination of whether quizartinib is indicated:

12.6.1. 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 is indicated.

12.6.2. 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 is not indicated.

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

12.7. Fail Detail codes are provided in the LeukoStrat CDx *FLT3* Software report; repeat the run or retest samples according to instructions in section 13: *Retesting*.

13. Retesting

13.1. Invalid Runs

13.1.1. 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.

13.1.2. 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.

13.2. Invalid Extraction Control within Valid Runs

13.2.1. 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.

13.3. Invalid Samples within Valid Runs

13.3.1. 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.

13.4. Fail Details and Retesting

13.4.1. 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.

13.4.2. The hierarchy of retesting is

1) Invalid ITD or TKD Positive Control (PC) or No Template Control (NTC) (see section 13.1)
2) Invalid Extraction Control (EC) within a valid run (see section 13.2)
3) Invalid samples within a valid run (see section 13.3)

Figure 8 diagrams the hierarchy of retesting.

13.4.3. 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.

13.4.3.1. 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.

13.4.3.2. 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.

13.4.4. 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.

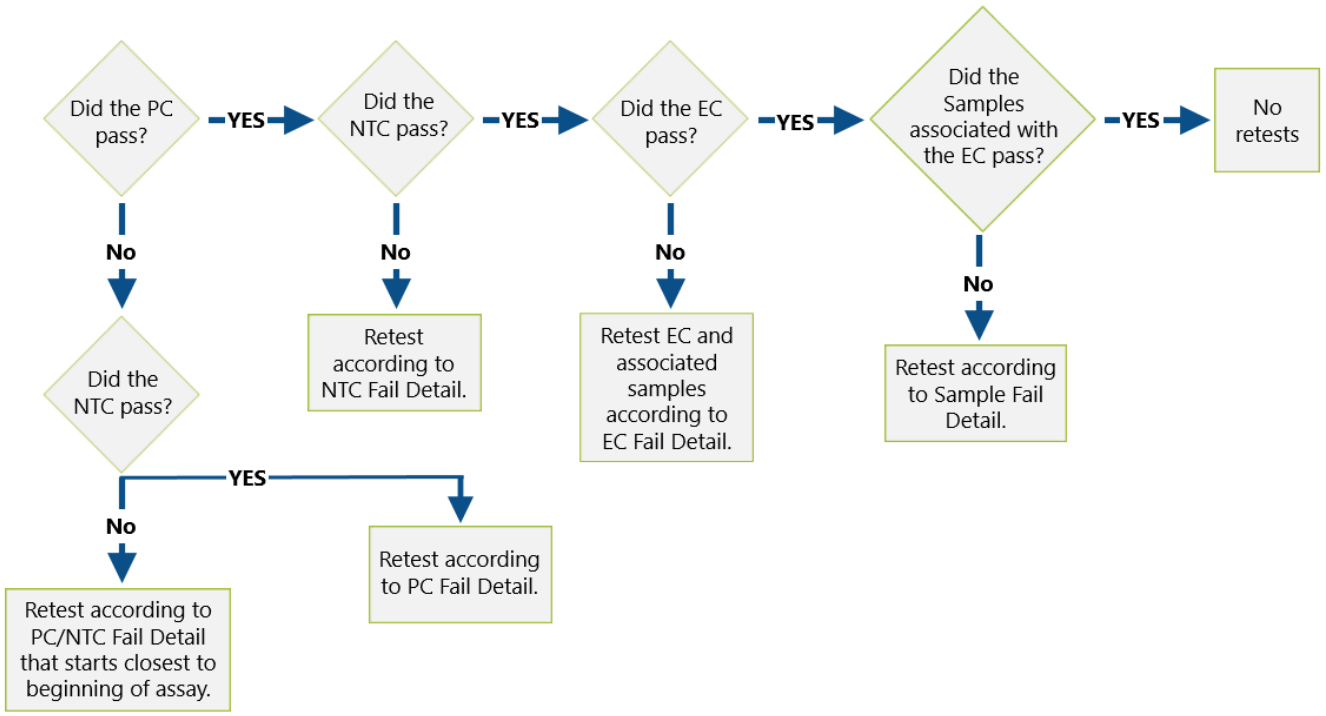


Figure 8. Retest hierarchy diagram

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
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

ITD 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/Pro
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.	10.10. *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.	10.12. *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.	10.12. *Capillary Electrophoresis Detection* 10.9. *Quantification and Dilution of DNA* 10.10. *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.	10.12. *Capillary Electrophoresis Detection* 10.2. *Sample Processing Preparation*

Table 13: Retest codes

Retest Code	Description	Retest Starting Point
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 13.6 *Dye Shift*). If the same Fail Detail (IR07) occurs a second time, report the sample as Invalid.	10.12. *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.	10.11. *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.	10.11. *Restriction Digest (TKD Mutation Only)* 10.2. *Sample Processing Preparation*
Q-Amp	Repeat beginning at quantification of Extraction Control(s), using previously made DNA sample dilutions.	10.9. *Quantification and Dilution of DNA* 10.10. *Amplification* (Samples)
Quant	Repeat beginning at quantification of all sample(s) and associated Extraction Control(s).	10.9. *Quantification and Dilution of DNA* 10.10. *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.	10.9. *Quantification and Dilution of DNA* 10.2. *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.	10.12. *Capillary Electrophoresis Detection* 10.9. *Quantification and Dilution of DNA* (Extraction Control(s)) 10.10. *Amplification* (Samples, PC) 10.2. *Sample Processing Preparation*

13.5. Multiple Failures within a Run

13.5.1. 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.

13.5.2. Additional troubleshooting actions may include the following items:

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

13.5.2.2. 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	

Table 14: Retesting, Multiple Failures within a Run

Sample Type	Fail Detail	Retest Code
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	CE
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.	10.10. *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.	10.12. *Capillary Electrophoresis Detection*
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.	10.12. *Capillary Electrophoresis Detection* 10.10. *Amplification*
CE-SS	Repeat beginning at capillary electrophoresis, using a new preparation of size standard solution.	10.12. *Capillary Electrophoresis Detection*

13.6. Dye Shift

13.6.1. 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.

14. Limitations of Procedure

14.1. 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.

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

14.3. 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, will not be reported as insertions.

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

14.4.1. 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.

14.4.2. 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.

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

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

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

14.7. 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.

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

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

15. Expected Values

15.1. Expected Size of Amplified Products

15.1.1. The amplicon sizes listed were determined using a 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.

16. Non-Clinical Performance Evaluation

16.1. All Evaluable Set:

The accuracy of the LeukoStrat CDx *FLT3* Mutation Assay was determined by comparing the results of the LeukoStrat CDx *FLT3* Mutation Assay to a validated high throughput sequencing method using specimens from the clinical trial. The samples for the method comparison study were a subset of the *FLT3* bridging study samples which included all available and evaluable *FLT3* mutation positive (CTA+) samples and approximately 300 *FLT3* mutation negative (CTA-) samples. The negative sample subset was selected by a randomization algorithm with the proportion from each CTA laboratory test site matching the proportion from that site in the overall A2301 study. After accounting for specimens with valid results, 505 CTA+ specimens were included and 263 CTA- specimens for a total of 768 patient specimens. Four of these contained low DNA quantity and were tested on deviation. Of the 764 results, 487 were *FLT3* positive by both assays and 230 were negative by both as summarized in Table 17. Agreement with and without the invalid results is shown in Table 18.

Table 17: Concordance between CDx and High throughput Sequencing for All Samples

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

Table 18: Agreement between CDx and High throughput Sequencing

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

***FLT3*-ITD:** The ITD detected population refers to the samples that harbor only ITD mutations based on Sequencing. Among the 378 ITD samples, 64% showed only one (1) ITD variant with the remaining containing multiple ITD mutations. The ITD insert length ranged from 3 bp to 209 bp. Most samples with ITD mutations were of insert lengths less than 100 bp (>85%). Thirty-seven (37) of the ITD samples contained insert lengths greater than or equal to 100 bp. The size distribution of the ITDs is shown in Figure 9.

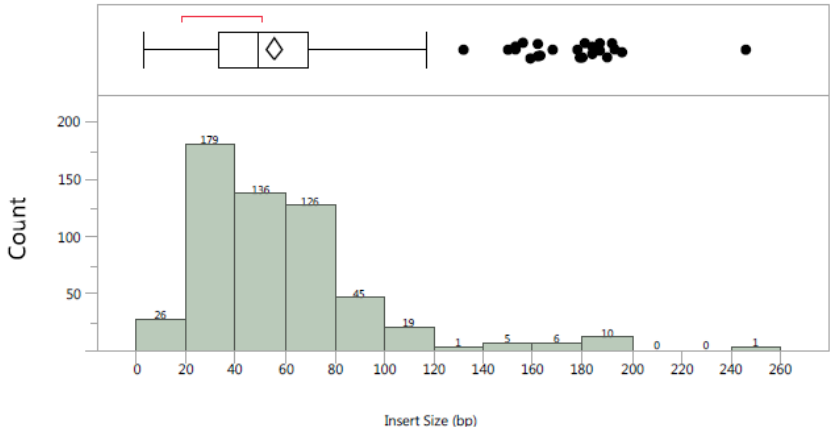


Figure 9: Distribution of ITD Insert Sizes by CDx (381 Positive Samples, N Insert Sizes = 554, Mean=55.6).

Nine patients failed to yield valid ITD results with the CDx. There were 57 discordant results among the 764 samples analyzed. Of the 57 discordant results, 50 showed low variant fraction reads by Sequencing and the CDx reported mutation negative based on the clinical cut-off (SR=0.05). The point estimates of PPA, NPA and OPA are 86.8%, 97.3% and 91.4%, respectively including the CDx invalids (Table 19). Without the CDx invalids, the PPA, NPA and OPA are at or above 88%.

Table 19: Agreement on ITD between CDx and Sequencing for *FLT3*-ITD results

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

***FLT3*-TKD:** The TKD detected population refers to samples that harbor only TKD mutations, based on high throughput sequencing. Among the 94 TKD samples, 79% contained one (1) TKD variant (substitution or deletion) while 20/94 (21%) contained two TKD variants. As expected, the single nucleotide substitution at codon D835 was the predominant mutation, mainly as D835Y. The D835H, D835V and I836S mutations were also observed at lower prevalence. Thirteen percent (13%) of the TKD positive samples contained the deletion variant at I836 as either a deletion only or deletion plus substitution.

137 (92.6%) of 148 samples, identified as positive for a TKD mutation by sequencing, were identified as TKD positive by the CDx assay. 611 (98.2%) of the 616 samples identified as TKD negative by sequencing, were TKD negative by the CDx assay. 8 patient samples yielded an invalid TKD result by the CDx and 8 of the 764 samples tested were discordant. The 8 discordant results showed low variant fraction reads by high throughput sequencing and the SR for the CDx reported mutation negative result found to be below the assay cut-off. Results for TKD agreement with and without invalids are summarized in Table 20.

Table 20: Agreement on TKD between CDx and Sequencing for *FLT3*-TKD

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

Results were analyzed separately for peripheral blood and bone marrow and demonstrated to be comparable.

16.2. 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.

16.3. Analytical Sensitivity

16.3.1. 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. Additional cell lines containing the D835 mutation and the I836 TKD mutation were 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 (5) 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 21. 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 21: 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	-
		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.

16.3.2. 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:

16.3.2.1. 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%.

16.3.2.2. 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%.

16.3.2.3. 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%.

16.3.2.4. 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%.

16.3.2.5. 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%.

16.3.2.6. Conversion of AR values to % Mutant is shown in the following Table 22.

Table 22: 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

16.4. Precision

16.4.1. The precision of the LeukoStrat CDx *FLT3* Mutation Assay was determined by three operators independently testing 10 replicates each of ITD mutation samples with inserts ranging in size from 21 bp to 126 bp and TKD mutation samples. The 10 replicates were tested in batches of two, a total of five separate times.

16.4.2. 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%.

16.4.3. 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%.

16.5. Multiple Site Precision and Reproducibility

16.5.1. The reproducibility of the LeukoStrat CDx FLT3 Mutation Assay was determined at three laboratory sites. A 21-member panel comprised of four cell line DNA and 17 contrived clinical DNA samples spanning a variety of ITD and TKD Mutation types was tested. 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 194 bp insert, TKD clinical DNA with a TKD D835 substitution and a TKD I836 deletion, and *FLT3* negative clinical DNA. Every clinical panel member was a mixture of DNA derived from peripheral blood and bone marrow. Clinical samples (excluding True Negative panel members) were tested at three signal ratio levels – High Negative (HN), Low Positive (LP), and Moderate Positive (MP). Each cell line DNA sample was tested at two signal ratio levels – HN and LP, resulting in four panel members. Two panel members were made from the clinical negative DNA. A total of 21 panel members were tested by each site. The panel representation is displayed in Table 23.

Table 23: Panel Members evaluated in the Multi-Site Reproducibility Study

Panel Type	Panel Description	Panel Composition	SR Level	Mutation Type	Target ITD SR	Target TKD SR
DNA: Cell Line	HN Medium ITD	126 bp cell line / *FLT3* Neg cell line	HN	ITD insert 126 bp	0.02 – 0.03	NA
DNA: Cell Line	LP Medium ITD	126 bp cell line / *FLT3* Neg cell line	LP	ITD insert 126 bp	0.07 – 0.11	NA
DNA: Cell Line	HN Long ITD	279 bp cell line / *FLT3* Neg cell line	HN	ITD insert 279 bp	0.02 – 0.03	NA
DNA: Cell Line	LP Long ITD	279 bp cell line / *FLT3* Neg cell line	LP	ITD insert 279 bp	0.07 – 0.11	NA
DNA: Clinical	HN Short ITD	ITD Pos Clin: 3 – 21 bp / Neg Clin	HN	ITD insert 6 bp	0.02 – 0.03	NA
DNA: Clinical	LP Short ITD	ITD Pos Clin: 3 – 21 bp / Neg Clin	LP	ITD insert 6 bp	0.07 – 0.11	NA
DNA: Clinical	MP Short ITD	ITD Pos Clin: 3 – 21 bp / Neg Clin	MP	ITD insert 6 bp	0.12 – 0.20	NA
DNA: Clinical	HN Medium ITD	ITD Pos Clin: 24 – 123 bp / Neg Clin	HN	ITD insert 69 bp	0.02 – 0.03	NA
DNA: Clinical	LP Medium ITD	ITD Pos Clin: 24 – 123 bp / Neg Clin	LP	ITD insert 69 bp	0.07 – 0.11	NA
DNA: Clinical	MP Medium ITD	ITD Pos Clin: 24 – 123 bp / Neg Clin	MP	ITD insert 69 bp	0.12 – 0.20	NA
DNA: Clinical	HN Long ITD	ITD Pos Clin: 129 bp – 276 bp / Neg Clin	HN	ITD insert 192 bp	0.02 – 0.03	NA
DNA: Clinical	LP Long ITD	ITD Pos Clin: 129 bp – 276 bp / Neg Clin	LP	ITD insert 192 bp	0.07 – 0.11	NA
DNA: Clinical	MP Long ITD	ITD Pos Clin: 129 bp – 276 bp / Neg Clin	MP	ITD insert 192 bp	0.12 – 0.20	NA
DNA: Clinical	HN D835 TKD	D835 Pos Clin / Neg Clin	HN	TKD D835	NA	0.02 – 0.03
DNA: Clinical	LP D835 TKD	D835 Pos Clin / Neg Clin	LP	TKD D835	NA	0.07 – 0.11
DNA: Clinical	MP D835 TKD	D835 Pos Clin / Neg Clin	MP	TKD D835	NA	0.12 – 0.20
DNA: Clinical	HN I836 TKD	I836 Pos Clin / Neg Clin	HN	TKD I836	NA	0.02 – 0.03
DNA: Clinical	LP I836 TKD	I836 Pos Clin / Neg Clin	LP	TKD I836	NA	0.07 – 0.11

Table 23: Panel Members evaluated in the Multi-Site Reproducibility Study

Panel Type	Panel Description	Panel Composition	SR Level	Mutation Type	Target ITD SR	Target TKD SR
DNA: Clinical	MP I836 TKD	I836 Pos Clin / Neg Clin	MP	TKD I836	NA	0.12 – 0.20
DNA: Clinical	ITD Negative	Pooled True Neg Clinical DNA	NA	Negative	0	0.00 – 0.01
DNA: Clinical	TKD Negative	Pooled True Neg Clinical DNA	NA	Negative	0	0.00 – 0.01

16.5.1. 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.

16.5.2. The lower limits of the Clopper-Pearson 95% two-sided confidence intervals for the positive and negative percent agreements (PPA and NPA). Signal ratio (SR) variance components were determined for Site/Instrument, Operator, Day/Run, Kit Lot, and Repeatability (Random Error). Variance components were estimated in terms of standard deviation (SD) and %CV per Low and Moderate Positive panel members (PM). The Moderate Positive (MP) Medium 69 bp ITD and TKD D835 variance components were estimated with and without outliers. After the outliers were removed, the total %CV changed from 12.6% to 6% for MP Medium 69 bp ITD and from 13.4% to 5.4% for MP TKD D835.

16.5.3. The total %CV for all panel members except Long Insert ranged from 3.8% to 13.4% with the outliers and from 3.8% to 10.2% without the outliers. The Low Positive (LP) Long 279 bp ITD PM's total %CV was 32.6% which is expected. The Long 192 bp ITD PM's total %CV was 12.7% for the MP SR level and 20.4% for the LP SR level. The variability due to Sites was negligible. The %CV was less than 5%. The majority of variability was due to random error except for the MP Long 192 bp ITD PM. For this panel member, the variability due to Day/Run was the greatest. The results are summarized in Table 24 and Table 25.

Table 24: 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

Table 25: Call Agreement with Expected Results Across Sites

Panel Description	SR Level	3 Sites Combined % (n/N) Expected Results
Short 6 bp ITD	LP	100% (72/72)
	MP	100% (72/72)
	HN	100% (72/72)
Medium 69 bp ITD	LP	100% (72/72)
	MP	100% (72/72)
	HN	100% (72/72)
Medium 126 bp ITD	LP	100% (72/72)
	HN	97.2% (70/72)
Long 192 bp ITD	LP	95.8% (69/72)
	MP	100% (72/72)
	HN	100% (72/72)
Long 279 bp ITD	LP	93.1% (67/72)
	HN	100% (72/72)
D835 TKD	LP	100% (72/72)
	MP	100% (72/72)
	HN	100% (72/72)
I836 TKD	LP	100% (72/72)
	MP	100% (72/72)
	HN	100% (72/72)
ITD Negative	N/A	100% (72/72)
TKD Negative	N/A	100% (72/72)

16.5.4. Precision of Controls was assessed based on the use in the multi-site study and is shown in Table 26 below.

Table 26: ITD and TKD Positive Control Signal Ratio

PC	N	Mean	SD	Minimum	Maximum	%CV
ITD PC	47	0.093	0.007	0.08	0.11	7.7
TKD PC	36	0.096	0.012	0.06	0.11	12.1

16.6. Single Site: Operator-to-Operator Reproducibility (cell-lines)

16.6.1. 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%.

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

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

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

16.7. Single Site: Operator-to-Operator Reproducibility (clinical samples)

16.7.1. 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.

16.7.2. The total %CV of all mutation types and levels are shown in Table 27 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 27: Components of Variance per Mutation Type and Dilution Level

Sample ID	Mut Type	Dilution Level	Mean SR	SR Variation Due to			Total Variation	
				Operator / Instrument SD (%)	Run Day SD (%)	Random Error SD (%)	SD	% CV
S1	TKD I836	HN	0.03	0.000 (3.22%)	0.000 (0.00%)	0.002 (96.78%)	0.002	7.1
		LP	0.077	0.001 (2.60%)	0.000 (0.00%)	0.005 (97.40%)	0.005	5.9
		MP	0.132	0.002 (6.67%)	0.003 (17.43%)	0.005 (75.90%)	0.006	4.6
S2	TKD D835	HN	0.04	0.001 (7.13%)	0.000 (0.00%)	0.002 (92.87%)	0.002	5.3
		LP	0.08	0.002 (14.02%)	0.001 (2.47%)	0.004 (83.51%)	0.004	5.3
		MP	0.165	0.003 (16.28%)	0.000 (0.00%)	0.007 (83.72%)	0.007	4.2
S3	ITD 21 bp	HN	0.03	0.000 (0.00%)	0.000 (0.00%)	0.001 (100.0%)	0.001	5
		LP	0.074	0.000 (0.00%)	0.002 (8.08%)	0.005 (91.92%)	0.005	7.2
		MP	0.133	0.002 (14.46%)	0.000 (0.00%)	0.005 (85.54%)	0.006	4.4
S4	ITD 24 bp	HN	0.029	0.000 (0.00%)	0.000 (0.00%)	0.004 (100.0%)	0.004	15.2
		LP	0.07	0.000 (0.00%)	0.000 (0.92%)	0.004 (99.08%)	0.004	5.3
		MP	0.147	0.002 (8.20%)	0.001 (3.28%)	0.006 (88.52%)	0.007	4.5
S5	ITD 66 bp	HN	0.029	0.001 (4.28%)	0.000 (0.00%)	0.005 (95.72%)	0.005	16.1
		LP	0.083	0.000 (0.00%)	0.001 (1.13%)	0.007 (98.87%)	0.007	8
		MP	0.185	0.000 (0.00%)	0.000 (0.00%)	0.010 (100.0%)	0.01	5.3
S6	ITD 90 bp	HN	0.03	0.001 (5.15%)	0.000 (0.00%)	0.003 (94.85%)	0.003	10.1
		LP	0.091	0.004 (25.23%)	0.002 (8.42%)	0.007 (66.35%)	0.008	8.5
		MP	0.206	0.013 (44.26%)	0.005 (7.34%)	0.013 (48.40%)	0.019	8.5
S7	ITD 217 bp	HN	0.032	0.001 (0.90%)	0.002 (7.20%)	0.008 (91.90%)	0.009	27.2
		LP	0.079	0.013 (31.42%)	0.009 (14.86%)	0.017 (53.71%)	0.023	26.9
		MP	0.162	0.029 (36.75%)	0.015 (9.86%)	0.035 (53.39%)	0.047	27.2

16.8. Lot-to-Lot and Instrument-to-Instrument Reproducibility

16.8.1. 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.

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

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

16.9. Interfering Substances – Exogenous

16.9.1. No interference in the performance of the LeukoStrat CDx *FLT3* Mutation Assay was observed in the detection of ITD mutations sized 18 bp to 114 bp and TKD mutations of clinical samples in the presence of Na-Heparin (0.8 mg/mL).

16.9.2. No interference in the performance of the LeukoStrat CDx *FLT3* Mutation Assay was observed in the detection of the Assay Positive Controls in the presence of the wash buffer (10% v/v AW2) used during the DNA isolation process.

16.10. Interfering Substances – Endogenous

16.10.1. No interference in the performance of the LeukoStrat CDx *FLT3* Mutation Assay was observed in the detection of ITD mutations sized 18 bp to 114 bp and TKD mutations of clinical samples in the presence of lipids/triglycerides (5% v/v), hemoglobin (2mg/mL), protein (60 mg/mL) and bilirubin (0.19 mg/mL).

16.11. Interfering Substances – Treatment Drugs

16.11.1. No interference in the performance of the LeukoStrat CDx *FLT3* Mutation Assay was observed in the detection of ITD mutations sized 18 bp to 114 bp and TKD mutations of clinical samples in the presence of cytarabine (24 µg/mL) and daunorubicin (180 ng/mL).

16.12. Carryover and Cross Contamination

16.12.1. 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:

16.12.1.1. Carryover / Cross Contamination detected was 0%.

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

16.13. DNA Input

16.13.1. 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)

16.13.2. 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%.

16.13.3. 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.

16.14. Validation of EDTA and Na-Heparin Anticoagulants Blood Collection Tubes

16.14.1. The purpose of this study was to demonstrate the equivalent performance of the assay when either EDTA or Na-Heparin is used as an anticoagulant. 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.

16.14.2. Low positive and moderate positive samples resulted in 100% positive replicates in both EDTA and Na-Heparin. All panel members were 100% concordant (24/24 replicates) for samples prepared in either Na-heparin or EDTA. SRs are summarized descriptively by mean, standard deviation (SD), minimum (min), maximum (max), median and the coefficient of variation (%CV) for each panel member in Table 28. The EDTA SR %CV ranged from 6.7% to 17.8% and the Na-Heparin SR %CV ranged from 7.5% to 16.3% which is consistent with the data observed in the precision studies.

Table 28: Signal Ratio Descriptive Statistics per Panel Member and Anticoagulant

Sample Type	SR Level	Anticoagulant	SR Mean (SD)	95% CI	SR Median	SR Min – Max	%CV
Short ITD	HN	EDTA	0.023 (0.005)	0.021 - 0.025	0.02	0.020 - 0.030	N/A
		Na-Heparin	0.023 (0.005)	0.020 - 0.025	0.02	0.010 - 0.030	N/A
	LP	EDTA	0.098 (0.017)	0.091 - 0.105	0.11	0.070 - 0.120	17.0
		Na-Heparin	0.067 (0.008)	0.064 - 0.070	0.07	0.050 - 0.080	12.0
	MP	EDTA	0.151 (0.019)	0.143 - 0.158	0.16	0.122 - 0.180	12.3
		Na-Heparin	0.138 (0.010)	0.133 - 0.142	0.14	0.120 - 0.156	7.5
Medium ITD	HN	EDTA	0.023 (0.005)	0.021 - 0.025	0.02	0.020 - 0.030	N/A
		Na-Heparin	0.027 (0.005)	0.025 - 0.029	0.03	0.020 - 0.030	N/A
	LP	EDTA	0.083 (0.007)	0.079 - 0.086	0.08	0.070 - 0.100	8.9
		Na-Heparin	0.083 (0.011)	0.078 - 0.088	0.08	0.070 - 0.110	13.5
	MP	EDTA	0.111 (0.007)	0.108 - 0.114	0.11	0.100 - 0.120	6.7
		Na-Heparin	0.113 (0.012)	0.108 - 0.118	0.12	0.090 - 0.130	10.9
Long ITD	HN	EDTA	0.018 (0.010)	0.014 - 0.022	0.02	0.000 - 0.030	N/A
		Na-Heparin	0.021 (0.008)	0.017 - 0.024	0.02	0.000 - 0.030	N/A
	LP	EDTA	0.072 (0.011)	0.067 - 0.076	0.07	0.050 - 0.090	15.8
		Na-Heparin	0.086 (0.012)	0.081 - 0.091	0.08	0.070 - 0.110	13.7
	MP	EDTA	0.112 (0.020)	0.104 - 0.121	0.11	0.080 - 0.160	17.8
		Na-Heparin	0.126 (0.012)	0.121 - 0.132	0.13	0.100 - 0.143	9.9

Table 28: Signal Ratio Descriptive Statistics per Panel Member and Anticoagulant

Sample Type	SR Level	Anticoagulant	SR Mean (SD)	95% CI	SR Median	SR Min – Max	%CV
TKD D835	HN	EDTA	0.023 (0.005)	0.021 - 0.025	0.02	0.020 - 0.030	N/A
		Na-Heparin	0.028 (0.004)	0.026 - 0.030	0.03	0.020 - 0.030	N/A
	LP	EDTA	0.077 (0.010)	0.073 - 0.081	0.08	0.060 - 0.100	12.6
		Na-Heparin	0.085 (0.014)	0.079 - 0.091	0.08	0.070 - 0.110	16.3
	MP	EDTA	0.165 (0.015)	0.158 - 0.171	0.16	0.137 - 0.193	9.1
		Na-Heparin	0.183 (0.019)	0.175 - 0.192	0.18	0.156 - 0.221	10.6
TN ITD	TN	EDTA	0.000 (0.000)	N/A	0.00	0.000 - 0.000	N/A
		Na-Heparin	0.000 (0.000)	N/A	0.00	0.000 - 0.000	N/A
TN TKD	TN	EDTA	0.006 (0.005)	0.004 - 0.008	0.01	0.000 - 0.010	N/A
		Na-Heparin	0.005 (0.005)	0.003 - 0.007	0.01	0.000 - 0.010	N/A

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

16.15. Density Gradient Media Validation

16.15.1. 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. The assay was challenged by testing the ability of the density gradient media (DGM) to isolate small populations (or percentages) of mutant cells. 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). The contrived panel members for the 21bp ITD insert and the TKD cell lines contained a mutant content up to 5% cells. The long ITD insert had a mutant content up to 30% cells, due to a known Assay limitation as a result of PCR bias for long insert lengths. 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.

16.15.2. 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). The mutant content was low for all panel members and therefore only a small portion of the panel member replicates were expected to yield positive SRs (above the clinical cutoff). Positive calls per DGM2 were within 2.5% of positive calls by DGM1 and positive calls per DGM3 were within 1.2% of positive calls by DGM1; therefore meeting the requirement of the overall positive calls being within 10% of DGM1.

16.15.3. Using DGM1 as the reference, the point estimates for PPA were 93.3% and 96.7% for DGM2 and DGM3, respectively. The point estimates for NPA using DGM1 as reference were 100% for both DGM2 and DGM3.

16.15.4. 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.

16.16. 3500xL Dx Genetic Analyzer Validation

16.16.1. The purpose of this study was to show equivalency between the 3500xL Genetic Analyzer (3500xL) and the 3500xL Dx Genetic Analyzer (3500xL Dx) instrument. The clinical DNA samples tested spanned a range of ITD insertions (24 bp, 48 bp, and 180 bp), a TKD D835 substitution, a TKD I836 deletion, and *FLT3* negative samples. All samples, excluding the negative sample, were tested at three signal ratio levels: high negative, low positive, and moderate positive (resulting in 16 panel members).

16.16.2. Each panel member was tested in triplicate over six non-consecutive days on two 3500xL instruments. The panel members were tested using all combinations of two lots of LIZ Size Standard and POP7 Polymer on two 3500xL Dx instruments. There were a total of 36 replicates per 3500xL and 72 replicates per 3500xL Dx per panel member.

16.16.3. The signal ratio %CV for positive panel members ranged from 3.4% to 10.5% on the 3500xL Dx and 3.3% to 10.0% on the 3500xL, well under the required 25% CV. The lower limits of the Clopper-Pearson 95% two-sided confidence intervals for the positive and negative percent agreements per 3500xL Dx were 97.2% or greater, which met the higher than 90% criterion.

16.16.4. All study acceptance criteria were met which validates the use of the 3500xL Dx Genetic Analyzer for use in the LeukoStrat CDx *FLT3* Mutation Assay.

17. Clinical Performance Evaluation

17.1. IVS-002-001 Pivotal Bridging Study (RATIFY Clinical Trial)

17.1.1. Pivotal Bridging Study Overview (IVS-002-001)

17.1.1.1. To support the safety and efficacy assessment of the LeukoStrat CDx *FLT3* Mutation Assay, clinical agreement was required to be demonstrated between samples with *FLT3* status determined from the A2301 Clinical Trial Assay (CTA) and the LeukoStrat CDx *FLT3* Mutation Assay in the intent-to-test population. This pivotal Bridging Study for the LeukoStrat CDx *FLT3* Mutation Assay, corresponds to the Phase III CPKC412A2301 (A2301, CALBG 10603, RATIFY) clinical study of midostaurin in newly diagnosed AML patients with *FLT3* mutations. The LeukoStrat CDx *FLT3* Mutation Assay is intended to assist physicians in making treatment decisions for their AML patients with *FLT3* Mutations.

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

17.1.2. Study Objectives (IVS-002-001)

17.1.2.1. Primary objectives of the study were to 1) establish agreement with respect to selection of *FLT3* mutant patients between the A2301 CTA and the LeukoStrat CDx *FLT3* Mutation Assay by assessing the overall, positive and negative percent agreement between the two assays and 2) to estimate midostaurin efficacy in the LeukoStrat CDx *FLT3* Mutation Assay positive population on both overall survival (OS).

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

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

17.1.3. Patient Population (IVS-002-001)

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

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

17.1.4. Selection of Patients and Aliquots for *FLT3* CDx Testing (IVS-002-001)

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

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

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

17.1.5. Safety Analysis (IVS-002-001)

17.1.5.1. 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 risk.

17.1.6. Effectiveness (IVS-002-001)

17.1.6.1. Primary CDx Clinical Validation analyses were performed using samples from 1058 AML patients from the A2301 study population. The LeukoStrat *FLT3* Mutation CDx Assay demonstrated agreement to the CTA, similar efficacy to A2301 and accuracy compared to high throughput sequencing (Table 29).

17.1.6.1.1. The primary analysis demonstrated:
The CTA/CDx agreement for *FLT3* status was high (>97%), with positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) well above the 90% acceptance criteria for positive and negative agreement.

Table 29. Overall Agreement Table between CDx and CTA

FLT3 CDx	*FLT3* CTA	
	+	-
+	489	8
-	9	540
Invalid	5	7
Total	503	555

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

Agreement (95% CI) including the CDx Invalid samples are:
- PPA 97.2% (95.4%, 98.5%)
- NPA 97.3% (95.6%, 98.5%)
- OPA 97.3% (96.1%, 98.2%)

The CTA/CDx agreement for the individual ITD and TKD tests (PPA, NPA, OPA) was above 90% (Table 30 and Table 31).

Table 30. Agreement Table between ITD CDx and ITD CTA

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

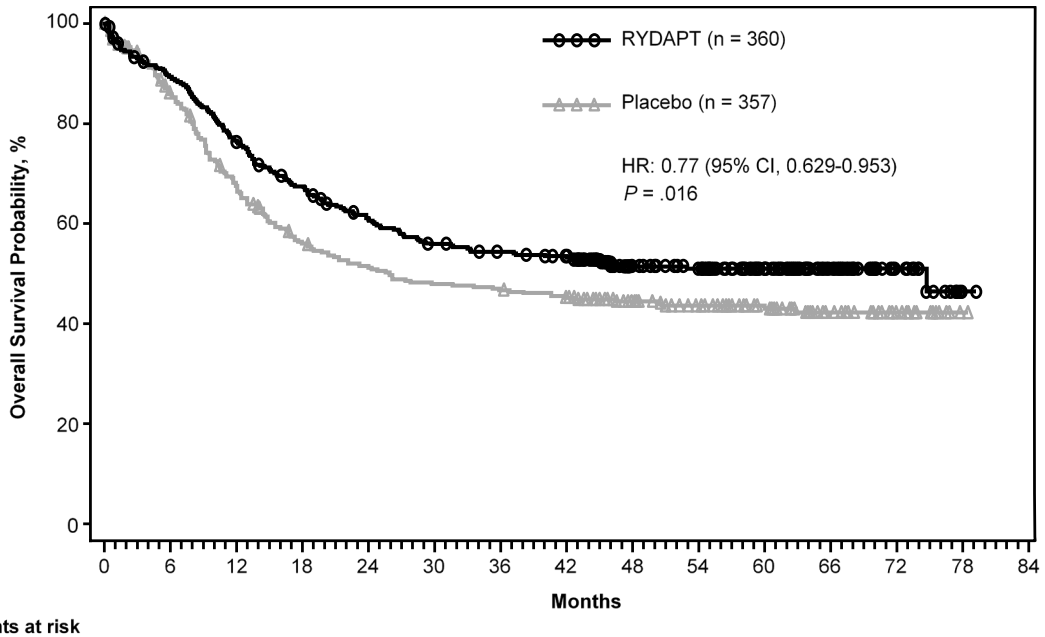
- Invalid means that a sample was tested on the CDx assay but failed to return a valid result.

Table 31. Agreement Table between TKD CDx and TKD CTA

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

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

In the clinical trial, efficacy was established on the basis of overall survival (OS) using the CTA, measured from the date of randomization until death by any cause. The primary analysis was conducted after a minimum follow-up of approximately 3.5 years after the randomization of the last patient. RYDAPT plus standard chemotherapy was superior to placebo plus standard chemotherapy in OS (HR 0.77; 95% CI 0.63, 0.95; 2 sided p=0.016) (Figure 10). Because survival curves plateaued before reaching the median, median survival could not be reliably estimated.



Patients at risk

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

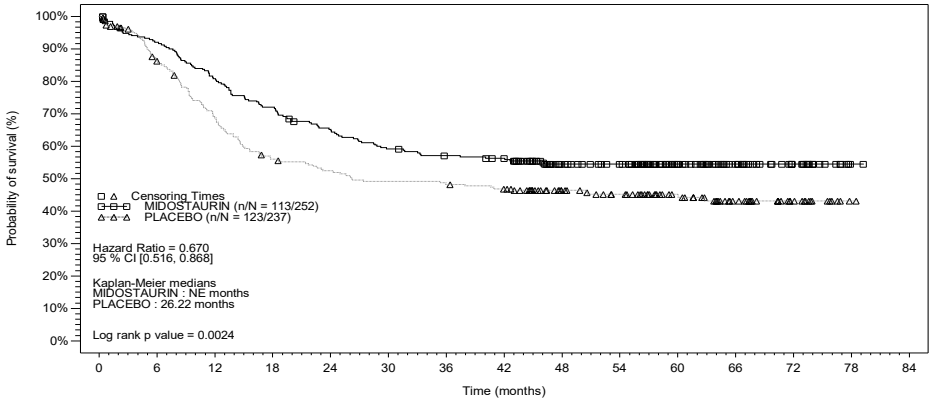
Figure 10. Kaplan-Meier for overall survival in the A2301 Trial in the CTA+ population.

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

Midostaurin efficacy on overall survival in the CDx-positive population was estimated. Efficacy determined in the (CTA+, CDX+) population was similar between the overall A2301 clinical trial results and the CDx tested subset and for overall survival with non-censoring at stem cell transplant (See Figure 10 and Figure 11, respectively). Hazard ratio (95% CI) outcomes for OS were 0.67 (0.52, 0.87) vs A2301 OS 0.77 (0.63, 0.95).

CPKC412A2301/CALGB10603 - Bridging Study

Figure 12-8
Kaplan-Meier curve for overall survival in the (CTA+, CDx+) population - model 2, non-censored for stem cell transplantation (PAS)



No. of patients still at risk

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

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

/vob/CPKC412A/pool/pool_003/report/pgm_eff/f_km_13_8.sas - 26MAY2016 04:27 Final Version

Figure 11. Kaplan-Meier for overall survival in the A2301 Trial in the CTA+, CDx+ Population.

17.1.6.1.3. The secondary and other analyses demonstrated:

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

17.1.6.1.3.2. Peripheral blood and bone marrow concordance (PPA and NPA) was greater than 95%, indicating both sample specimen types can be used for patient diagnosis (Table 32 and Table 33).

Table 32. Agreement Table between Peripheral Blood and Bone Marrow

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

Table 33. Agreement between Peripheral Blood and Bone Marrow

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

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

17.1.7. Conclusions (IVS-002-001)

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

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

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

17.2.1. Study Overview (IVS-056-001)

17.2.1.1. 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 gilteritinib/XOSPATA 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.

17.2.2. Study Objective (IVS-056-001)

17.2.2.1. Determine the efficacy of gilteritinib (XOSPATA) therapy as assessed by the rate of complete remission and complete remission with partial hematological recovery (CR/CRh) in subjects with *FLT3*-mutated AML who are refractory to or have relapsed after first-line AML therapy.

17.2.3. Patient Population (IVS-056-001)

17.2.3.1. Specimens from 485 subjects were screened with the LeukoStrat CDx *FLT3* Mutation Assay as of the cut-off date. 138 patients in the gilteritinib arm were included in the first interim analysis. 121 subjects had ITD mutations, 12 had TKD mutation and 5 had both ITD and TKD. Subjects were enrolled in the study based on identification of *FLT3* mutations in a BM or PB sample. The clinical cut-off of the test for the clinical study was set at 0.05 (mutant : WT signal ratio).

17.2.4. Safety Analysis (IVS-056-001)

17.2.4.1. 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.

17.2.5. Effectiveness (IVS-056-001)

17.2.5.1. Median duration of response in subjects with response of CR/CRh was 4.6 months. In the group of subjects with best response of CR, the median duration of response was 8.6 months. In the group of subjects with response of CRh, the median duration of response was 2.9 months. For patients who achieved a CR/CRh, the median time to first response was 3.6 months (range, 0.9 to 9.6 months). The CR/CRh rate was 29 of 126 in patients with *FLT3*-ITD or *FLT3*-ITD/TKD and 0 of 12 in patients with *FLT3*-TKD only. Efficacy results are provided in Table 34.

Table 34. Efficacy Results in Patients with Relapsed or Refractory AML

Remission Rate	ADMIRAL XOSPATA (120 mg daily) N=138
CR*/CRh† n/N (%)	29/138 (21)
95% CI‡	14.5, 28.8
Median DOR§ (months)	4.6
Range (months)	0.1 to 15.8¶
CR* n/N (%)	16/138 (11.6)
95% CI‡	6.8, 18.1
Median DOR§ (months)	8.6
Range (months)	1 to 13.8
CRh† n/N (%)	13/138 (9.4)
95% CI‡	5.1, 15.6
Median DOR§ (months)	2.9
Range (months)	0.1 to 15.8¶

CI: confidence interval; NE: not estimable; NR: not reached; Only responses prior to HSCT were included in response rate.

*CR was defined as an absolute neutrophil count ≥1.0 x 10^9/L, platelets ≥100 x 10^9/L, normal marrow differential with <5% blasts, must have been red blood cells, platelet transfusion independent and no evidence of extramedullary leukemia.

†CRh was defined as marrow blasts <5%, partial hematologic recovery absolute neutrophil count ≥0.5 x 10^9/L and platelets ≥50 x 10^9/L, no evidence of extramedullary leukemia and could not have been classified as CR.

‡The 95% CI rate was calculated using the exact method based on binomial distribution.

§DOR was defined as the time from the date of either first CR or CRh until the date of a documented relapse of any type. Deaths were counted as events.

¶Response was ongoing.

17.2.6. Conclusions

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

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

17.3.1. Pivotal Bridging Study Overview (IVS-062-005)

17.3.1.1. 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.

17.3.1.2. The LeukoStrat CDx *FLT3* Mutation Assay has been developed by Invivoscribe as a companion diagnostic to be used as an aid in the assessment of AML patients for whom 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.

17.3.2. Study Objectives (IVS-062-005)

17.3.2.1. 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.

17.3.2.2. 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).

17.3.3. Patient Population (IVS-062-005)

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.

17.3.4. Safety Analysis (IVS-062-005)

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.

17.3.5. Effectiveness (IVS-062-005)

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

17.3.5.1.1. 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 35: 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%)

17.3.5.1.2. 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.

17.3.6. Conclusions (IVS-062-005)

17.3.6.1. 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.

18. References

1. Murphy KM, Levis M, Hafez MJ, Gieger T, Copper LC, Smith BD, Small D and Berg KD. Detection of *FLT3* Internal Tandem Duplication and D835 Mutations by a Multiplex Polymerase Change Reaction and Capillary Electrophoresis Assay. *Journal of Molecular Diagnostics,* 2003, 5:96-102.
2. Yamamoto, Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of *FLT3* in human hematologic malignancies. *Blood*, 2001, 97(8):2434-9.

19. Technical and Customer Service

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

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