Instructions for Use

FLT3 Mutation Assay

RUO

Identifies internal tandem duplications and D835 mutations in the *FLT3* gene.

RUO For RESEARCH USE ONLY. Not for use in diagnostic procedures.

Manufactured in U.S.A.

invivoscribe

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

Catalog#	Products	Quantity
REF 1-412-0010	*FLT3* Mutation Assay for Gel Detection	33 Reactions
REF 1-412-0031	*FLT3* Mutation Assay for ABI Fluorescence Detection	33 Reactions
REF 1-412-0020	*FLT3* Mutation Assay MegaKit for Gel Detection	330 Reactions
REF 1-412-0041	*FLT3* Mutation Assay MegaKit for ABI Fluorescence Detection	330 Reactions

Table of Contents

1. Assay Use

This Research Use Only assay identifies *FLT3* mutations are useful for the study of:

- Identifying *FLT3* mutations in patients with AML.
- Discriminating between high and low risk patients.

FLT3 mutations portend a worse prognosis for patients with AML. Therefore patients testing positive for *FLT3* mutations may benefit from a more aggressive treatment regimen.

2. Summary and Explanation of the Test

2.1. Summary

Acute myeloid leukemia (AML) in general has a poor prognosis. Recent studies have described mutation of the *FLT3* (FMS-like tyrosine kinase 3) receptor to be the most important prognostic factor in AML, with *FLT3* mutants having a worse outcome and response to standard chemotherapeutic interventions. Accordingly, identification of an *FLT3* mutation in AML may indicate a need to reassess and modify standard treatment options.

The *FLT3* gene (aliases: *STK1*; *CD135*; *FLK-2*) contains 24 exons and spans at least 96 kb. *FLT3* is a receptor tyrosine kinase that is normally expressed on many cell types including hematologic stem cells.

All types of AML can have activating mutations in the *FLT3* gene. Mutation of the *FLT3* receptor, either by internal tandem duplication (ITD) of the juxtamembrane domain or by point mutation of the aspartic acid residue D835 in the activation loop of the kinase domain, causes constitutive activation of the *FLT3* receptor.

2.2. Background

Mutations in the FMS related tyrosine kinase 3 (*FLT3*) gene are the most common mutations found in acute myeloid leukemia (AML), occurring in approximately 30% of patients at the time of diagnosis, and are characterized by an aggressive phenotype with a high prevalence of relapse.[1,2,3] The most prevalent and clinically significant type of *FLT3* mutation is an internal tandem duplication (ITD) in the juxtamembrane domain.[4] Many clinical studies have found that *FLT3* ITD mutations are associated with higher concentrations of leukemic cells in both blood and bone marrow, increased incidence of relapse, and decreased overall survival. The second most common mutation type in the *FLT3* gene is a tyrosine kinase domain (TKD) point mutation in aspartate (D835) or isoleucine (I836). TKD mutations result in constitutive autophosphorylation and activation of *FLT3*.[5,6] TKD mutations have been linked to poor overall survival, but to a lesser extent as compared to ITD mutations.

3. Principles of the Procedure

3.1. Polymerase Chain Reaction (PCR)

FLT3 internal tandem duplication or length mutations are caused by duplication and insertion of a portion of the *FLT3* gene that includes the region in and around the juxtamembrane (JM) region of the *FLT3* gene. These mutations vary in both the location and the length of the inserted duplicated DNA sequence. ITD mutations result in constitutive autophosphorylation and activation of *FLT3*.[1] Wild-type *FLT3* alleles will amplify and produce a 330 bp product using this assay, while alleles that contain ITD mutations will produce a product that exceeds 330 bp.

FLT3 tyrosine kinase domain (TKD) mutations are caused by nucleic acid substitutions that result in a change in the amino acid sequence in this highly conserved catalytic center. TKD mutations, such as D835 and I836, result in constitutive autophosphorylation and activation of *FLT3*.[2] Wild-type alleles of the *FLT3* gene include an EcoRV restriction digest site. When a nucleic acid substitution occurs, the restriction digest recognition site disappears, and the EcoRV endonuclease is unable to identify and digest the DNA at this site. The *FLT3* target region is amplified using PCR and then an EcoRV restriction digest is performed. One of the PCR primers contains an EcoRV restriction site, so both wild type and mutant alleles are digested. The digestion pattern identifies loss of the normal gene sequence and ensures that digestion occurred. Wild type alleles of the *FLT3* gene yield digestion products of 80 bp whereas mutant alleles yield products of 130 bp from the original undigested amplicon product of 150 bp, please see Figure 1 for more details.

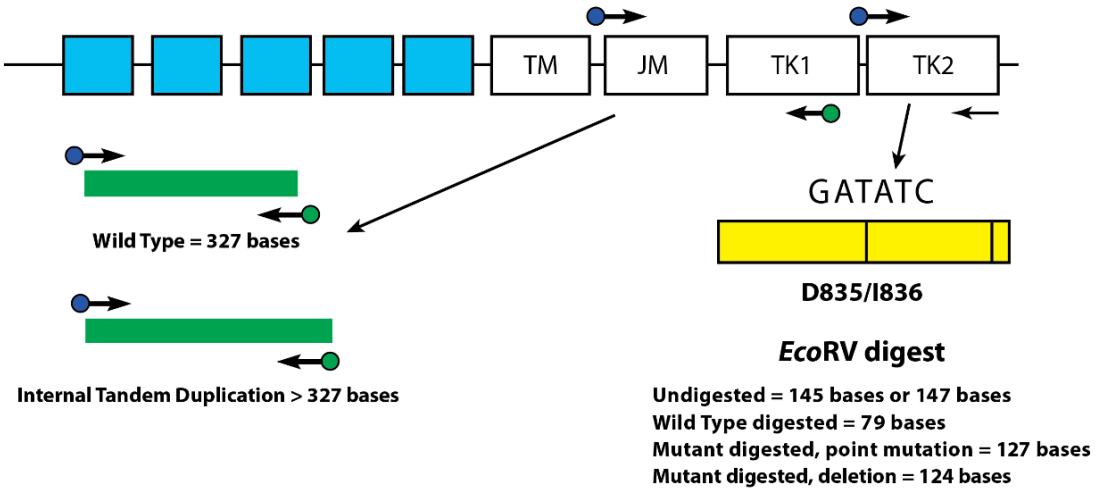

Figure 1. This figure shows a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target in and around the JM region for ITD or the activating loop of the 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.

This test kit includes three master mixes. The ITD and D835 master mixes target the juxtamembrane and kinase domain regions (respectively). The third master mix, the Specimen Control Size Ladder, targets multiple genes and generates a series of amplicons of 100, 200, 300, 400, and 600 bp to ensure that the quality and quantity of input DNA is adequate to yield a valid result.

3.2. Gel Detection

Gel electrophoresis, such as agarose gel electrophoresis or non-denaturing polyacrylamide gel electrophoresis (PAGE), is commonly used to resolve the different amplicon products based on their size, charge, and conformation. Since DNA is negatively charged, when an electrical potential (voltage) is applied across the gel containing PCR products, the electrical field causes the amplicons to migrate through the gel. Smaller DNA fragments are able to easily migrate through the gel matrix, whereas larger DNA fragments migrate more slowly. This causes a separation of the amplicon products based on size. Ethidium bromide or other DNA intercalating dyes can then be used to stain and detect these products in the gel.

3.3. Differential Fluorescence Detection

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

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog #	Description	Total Reactions
REF 1-412-0010	*FLT3* Mutation Assay for Gel Detection	33 Reactions
REF 1-412-0031	*FLT3* Mutation Assay for ABI Fluorescence Detection	33 Reactions
REF 1-412-0020	*FLT3* Mutation Assay MegaKit for Gel Detection	330 Reactions
REF 1-412-0041	*FLT3* Mutation Assay MegaKit for ABI Fluorescence Detection	330 Reactions

Table 2. Reagent Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Assay MegaKit # of Units	Storage Temp.
Master Mixes	2-412-001X*	***FLT3* ITD Master Mix** Multiple oligonucleotides targeting the *FLT3* gene in a buffered salt solution.	1500 µL	1	10	-85°C -65°C
	2-412-0020 and 2-412-0031	***FLT3* D835 Master Mix** Multiple oligonucleotides targeting the *FLT3* TKD region in a buffered salt solution.	1500 µL	1	10	
	2-096-002X*	**Specimen Control Size Ladder** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	10	
Positive Control DNA	4-088-0970	**IVS-0017 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	
	4-090-0010	**IVS-P001 Clonal Control DNA** 200 pg/mL of DNA in 1/10th TE solution	100 µL	1	5	
Negative Control DNA	4-092-0010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	5	

Note: There are no preservatives used during the manufacturing process of this kit.
***Note:** X = detection format code

4.2. Warnings and Precautions

- Use this assay kit as a system; do not substitute other manufacturers' reagents. Dilution, reducing amplification reactions or other deviations from this protocol may affect the performance of this test and/or nullify any limited sublicense that come with the purchase of these kits.
- 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.
- Adherence to the protocol will assure optimal performance and reproducibility. Ensure correct thermal cycler programs are used, as other programs may provide inaccurate/faulty data such as false-positive and false-negative results.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- Perform all laboratory procedures with standard personal protective equipment (gloves, laboratory coats and protective eyewear). 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.
- Use molecular biology grade water for the preparation of specimen DNA.
- Due to the high analytical sensitivity of this test, use extreme care to avoid any 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 any reagents suspected of contamination.
- To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to setting up PCR.
- Follow uni-directional workflow between separate work areas in the PCR laboratory: begin with master mix preparation, move to specimen preparation, then to amplification and finally to detection.
- Autoclaving does not eliminate DNA contamination. Perform pre- and post-PCR steps in separate spaces. Avoid taking paper and other materials from post-PCR into the pre-PCR space.
- Dedicate all pipettes, pipette tips and any equipment used in a particular area to that area of the laboratory.
- Decontaminate non-disposable items with 10% bleach and rinse with distilled water two separate times before returning them to the starting areas.
- Use sterile, disposable plastic ware whenever possible to avoid contamination

4.3. Storage and Handling

- Store the assay at **-85ºC to -65ºC** until ready to use.
- All reagents and controls must be thawed and vortexed or mixed thoroughly prior to use to ensure that they are completely resuspended.
- Due to high salt concentrations, PCR master mixes are sensitive to freeze/thaw cycles. Minimize the exposure of master mixes to freeze/thaw cycles.

If you have any questions, please contact the Invivoscribe technical staff. We would be happy to help you determine your optimal storage needs.

5. Instruments

5.1. Thermal cycler

- Use or Function: Amplification of DNA samples
- Suggested Instrument: Veriti™ Thermal Cycler or equivalent
- Performance Characteristics and Specification:
  - Minimum Thermal Range: 15°C to 96°C
  - Minimum Ramping Speed: 0.8°C/sec
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- See section 7.4. *Amplification* for thermal cycler program.

5.2. Electrophoresis Unit

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

5.3. UV Illumination Unit (for Gel Detection)

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

5.4. ABI Capillary Electrophoresis Instruments (for ABI Detection)

- Use or Function: Fragment detection and analysis
- Performance Characteristics and Specification:
  - The following capillary electrophoresis instruments will meet the performance needs for this assay:
    - ABI 310 Genetic Analyzer (1-capillary)
    - ABI PRISM® 373 DNA Sequencer
    - ABI PRISM 377 DNA Sequencer
    - ABI 3100 Avant Genetic Analyzer (4-capillaries)
    - ABI 3100 Genetic Analyzer (16-capillaries)
- Follow manufacturer's installation, operation, calibration and maintenance procedures.
- The ABI instrument used must be calibrated with appropriate Matrix Standards as outlined in section 7.2. *Materials Required (not provided).*
- Use the default settings for your polymer and capillary type.
- See section 7.6. *Detection* for more details.

6. Specimen Collection and Preparation

6.1. Precautions

Biological specimens from humans may contain potentially infectious materials. Handle all specimens in accordance with the OSHA Standard on Bloodborne Pathogens or BioSafety Level 2.

6.2. Interfering Substances

The following substances are known to interfere with PCR:

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

6.3. Specimen Requirements and Handling

This assay tests **genomic DNA** from the following sources:

- 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA. Ship at ambient temperature; OR
- Minimum 5 mm cube of tissue shipped frozen; or at room temperature or on ice in RPMI 1640; OR
- 2 µg of genomic DNA; OR
- Formalin-fixed paraffin embedded tissue or slides.

6.4. Sample Preparation

Using any method of DNA extraction, extract the genomic DNA from unknown samples. Resuspend DNA to final concentration of 100 µg – 400 µg per mL in TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0) or distilled water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNAs with the Amplification Control or Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

6.5. Available Template Amplification Controls

6.5.1. The **Amplification Control** master mix primers are available unlabeled for gel detection and labeled for differential fluorescence detection with a fluorescent dye (6-FAM). The 6-FAM label is detected as **BLUE** using the differential fluorescence software. The amplicons produced with this master mix are at 235 bp. Run the products of this master mix separately.

6.5.2. The **Specimen Control Size Ladder** master mix primers areavailable unlabeled for gel detection and labeled for differential fluorescence detection with a fluorescent dye (6-FAM). The 6-FAM label is detected as **BLUE** using the differential fluorescence software. The amplicons produced with this master mix are at ~100, 200, 300, 400, and 600 bp. Please note that the ~100 bp band is comprised of 84 bp and 96 bp bands; both of these bands co-migrate on a gel. Run the products of this master mix separately.

6.6. Sample Storage

Store genomic DNA at 2°C to 8°C or at -85°C to -65°C until use.

7. Assay Procedure

7.1. Materials Provided

Table 3. Materials Provided

Gel Detection Kits	
Catalog #	**Description**
REF 2-412-0010	*FLT3* ITD Master Mix - Unlabeled
REF 2-412-0020	*FLT3* D835 Master Mix - Unlabeled
REF 2-096-0020	Specimen Control Size Ladder - Unlabeled
REF 4-088-0970	IVS-0017 Clonal Control DNA
REF 4-090-0010	IVS-P001 Clonal Control DNA
REF 4-092-0010	IVS-0000 Polyclonal Control DNA

ABI Fluorescence Detection Kits	
Catalog #	**Description**
REF 2-412-0011	*FLT3* ITD Master Mix – 6FAM & HEX
REF 2-412-0031	*FLT3* D835 Master Mix – 6FAM
REF 2-096-0021	Specimen Control Size Ladder – 6FAM
REF 4-088-0970	IVS-0017 Clonal Control DNA
REF 4-090-0010	IVS-P001 Clonal Control DNA
REF 4-092-0010	IVS-0000 Polyclonal Control DNA

7.2. Materials Required (not provided)

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
Density Gradient Medium	Thermo Fisher Scientific®: • Ficoll-PAQUE Premium	45-001-752	N/A
Buffered Saline Solution	Thermo Fisher Scientific: • 1X PBS diluted from 10X PBS	70011-044	N/A
Growth Medium	Thermo Fisher Scientific: • RPMI 1640 with L-glutamine	11875-093	N/A
Dimethyl sulfoxide	Sigma: • DMSO Hybri-Max	D2650	N/A
Fetal Bovine Serum	Thermo Fisher Scientific: • HyClone™ Fetal Bovine Serum (U.S.), Characterized	SH3007103	N/A
DNA Polymerase	Roche: • EagleTaq DNA Polymerase	05206944190	N/A
Restriction Enzyme	New England Biolabs,: • EcoRV Restriction Endonuclease	R0195S	4,000 U @20 U/µL
Molecular Biology Grade or USP Water	N/A	N/A	DNase / RNase free
Calibrated Pipettes	N/A	N/A	Capable of accurately measuring volumes between 1 µL and 1000 µL.
Thermal cycler	Thermo Fisher Scientific: • Veriti Dx Thermal Cycler Bio-Rad: • MJ Research PTC-100 or PTC-200, PTC-220, PTC-240 Perkin-Elmer • PE 9600 or PE 9700	N/A	N/A
Vortex Mixer	N/A	N/A	N/A
PCR plates or tubes	N/A	N/A	Sterile

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
Filter barrier pipette tips	N/A	N/A	Sterile, RNase/DNase/ Pyrogen-free
Microcentrifuge tubes	N/A	N/A	Sterile
	Gel Detection		
Ethidium Bromide	Thermo Fisher Scientific: • UltraPure™ 10 mg/mL Ethidium Bromide	15585-011	N/A
Agarose Gels	Thermo Fisher Scientific: • NuSieve™ 3:1 Agarose, 125 g • MetaPhor™ Agarose, 125 g	BMA50090 BMA50180	
6% Polyacrylamide Gels	Thermo Fisher Scientific: • Novex® TBE Gels (6%, 12 well)	EC62652Box	N/A
TBE Running Buffer	Thermo Fisher Scientific: • Novex TBE Running Buffer (5X)	LC6675	Dilute 1:5 prior to use.
Gel Loading Buffer	Thermo Fisher Scientific: • 10X BlueJuice™ Gel Loading Buffer • Novex Hi-Density TBE Sample Buffer (5X)	10816-015 LC6678	N/A
100 bp DNA Ladder	Thermo Fisher Scientific: • TrackIt™ 100 bp DNA Ladder	10488-058	N/A
Gel Electrophoresis Unit	N/A	N/A	For agarose or polyacrylamide gels
	ABI Fluorescence Detection		
ABI Capillary Electrophoresis Instrument	Thermo Fisher Scientific: • ABI 310, 3100, 373, or 377 series	N/A	N/A
Hi-Di Formamide	Invivoscribe: • HI-Deionized Formamide Thermo Fisher Scientific: • Hi-Di™ Formamide	6-098-0011 4311320	N/A
Size Standards	Invivoscribe: • Hi-Di Formamide w/ROX size standards for ABI 310 • Hi-Di Formamide w/ROX size standards for ABI 3100 Thermo Fisher Scientific: • For ABI 3100 instruments: o GeneScan™ - 400HD [ROX]™	6-098-0051 6-098-0061 402985	N/A
Spectral Calibration Dye Sets	Thermo Fisher Scientific: • For ABI 3100 instruments: o DS-30 Matrix Standard Kit (Dye Set D) • For ABI 310 instruments: o NED Matrix Standard o And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX]	4345827 402996 401546	Dye set used to spectrally calibrate ABI instrument for use with 6FAM, HEX, NED and ROX
Polymer	Thermo Fisher Scientific: • POP-4™ Polymer: o POP-4 for 310 Genetic Analyzers o POP-4 for 3100/3100-Avant Genetic Analyzers	402838 4316355	N/A

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
Buffer	Thermo Fisher Scientific: o 10X Genetic Analyzer Buffer with EDTA	402824	Dilute 1:10 in sterile water before use

7.3. Reagent Preparation

- Unknown samples can be tested with a template amplification control (**Amplification Control** or **Specimen Control Size ladder)** master mix to ensure that no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result.
- Test all samples in **singlicate**.
- Test **positive**, **negative** and **no template** controls for each master mix.

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

7.3.2. In a containment hood or dead air box, remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each master mix).

- Aliquot volumes are 45 µL for each sample + 135 µL (3 x 45 µL) for the positive, negative, and no template (water) controls.
- Add an additional 20 µL to correct for pipetting errors.

7.3.3. Add the appropriate amount of Taq polymerase (0.25 µL @ 5 U/µl per 50 µL total PCR reaction volume) to each master mix and gently mix by inverting several times or gently vortexing.

- The master mixes are now ready for distribution to reaction tubes or plate and addition of sample.

7.4. Amplification

7.4.1. Aliquot 45 µL of the master mix/enzyme solutions into individual PCR wells or tubes.

7.4.2. Add 5 µL of sample or control DNA to the individual tubes or wells containing the respective master mix reactions, listed for each master mix in Table 5. Pipet up and down several times to mix.

Table 5. PCR Master Mix Testing Matrix

FLT3 ITD Master Mix:	*FLT3* D835 Master Mix:	Specimen Control Size Ladder:
Water IVS-0017 Clonal Control DNA IVS-0000 Polyclonal Control DNA Test Specimen DNA	Water IVS-P001 Clonal Control DNA IVS-0000 Polyclonal Control DNA Test Specimen DNA	Water IVS-0000 Polyclonal Control DNA Test Specimen DNA

7.4.3. Amplify the reactions using the following PCR program:

- Use the **calculated** option for temperature measurement with the PTC instruments.

Table 6. Thermal cycling conditions

Standard Program for AmpliTaq Gold or EagleTaq (RECOMMENDED)			
Step	**Temperature**	**Duration**	**Cycles**
1	95°C	**7 minutes**	1
2	94°C	30 seconds	35
3	55°C	30 seconds	
4	72°C	1 minute	
5	72°C	10 minutes	1
6	4°C	∞	1

Modified Program for AmpliTaq			
Step	**Temperature**	**Duration**	**Cycles**
1	95°C	**3 minutes**	1
2	94°C	30 seconds	35
3	55°C	30 seconds	
4	72°C	1 minute	
5	72°C	10 minutes	1
6	4°C	∞	1

7.4.4. Remove the amplification plate from the thermal cycler.

7.5. Digestion of *FLT3* D835 Master Mix reaction products

Note: The restriction digest is NOT performed on the ITD master mix.

7.5.1. Add the components in Table 7 to a clean microfuge tube or individual wells of a 96 well plate.

Table 7. Components per *FLT3* D835 Product Restriction Digest

Reagent	Volume per Reaction
FLT3 D835 Master Mix PCR reaction Products	10 µL
10X NEBuffer 3 (included with purchase of enzyme)	2 µL
EcoRV endonuclease (20 U/µL)	2 µL
Molecular Grade Water	16 µL
Total Volume	**30 µL**

7.5.2. Mix and place at 37ºC for 60 minutes.

7.5.3. Place reactions on ice or refrigerate until ready for detection

- If enzyme treatment is for less than the recommended 60 minutes, incomplete digestion may occur.

7.6. Detection

- Not all detection formats are available for all assays

Gel Detection – Agarose TBE Gels (RECOMMENDED)

7.6.1. A 2% MetaPhor or NuSieve 3:1 agarose/TBE gel is prepared.

7.6.2. Individually mix 20 µL from each amplification reaction with 4 µL of 6X gel loading buffer.

7.6.3. Load 20 µL of this mixture into separate wells of the gel, flanked by DNA size standards.

7.6.4. Detect products using ethidium bromide or an equivalent dye.

7.6.5. Photograph the gel and interpret the data.

Gel Detection – Polyacrylamide TBE Gels

7.6.6. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE) and 0.5X TBE running buffer.

7.6.7. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples.

7.6.8. Load 20 µL of mixture into wells of the gel.

7.6.9. Run gel at 110V for 2-3 hours or 40-50V overnight.

- Voltage and electrophoresis time depend on the PCR amplicon size, acrylamide gel thickness, and type of PCR equipment.
- Voltage and run time can be adapted accordingly.

7.6.10. Stain gels in 0.5 µg/mL Ethidium Bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.

7.6.11. Destain gels 2X in water for 5-10 minutes.

7.6.12. Visualize the gels with UV illumination.

7.6.13. Photograph the gel and interpret the data.

ABI Fluorescence Detection with ABI 310 & 3100 instruments

7.6.14. Combine 1 µL of the *FLT3* ITD and undigested *FLT3* D835 Master Mix products in a tube and add 20 µL of HI-Deionized Formamide containing ROX size standards. Mix well.

7.6.15. Combine 1 µL of the *FLT3* ITD and **digested** *FLT3* D835 Master Mix products in a tube and add 20 µL of HI-Deionized Formamide containing ROX size standards. Mix well.

7.6.16. Add 1 µL of reaction product from the template amplification control in a tube and add 10µl of HI-Deionized Formamide containing ROX size standards. Mix well.

7.6.17. Heat reaction products to 95ºC for 2 minutes then snap chill on ice for 5 minutes.

7.6.18. A **sample sheet** and **injection list** is made up for the samples.

- As the samples are run on the machine, they are fractionated, detected, and analyzed by the instrument.
- Data are automatically displayed as size and color specific peaks.

7.6.19. Review profile and controls, report results.

ABI Fluorescence Detection with ABI 373 & 377 instruments

7.6.20. PCR product dilution: initially dilute samples 1:10 in HI-Deionized Formamide or water.

- The dilution can be altered if the fluorescence signal is outside the optimal range.

7.6.21. Prepare the samples containing undigested *FLT3* D835 products by combining the reagents listed in Table 8 and mix well.

Table 8. Preparation of undigested *FLT3* D835 products for detection

Reagent	Volume per Reaction
FLT3 ITD Master Mix Product	2.0 µL
undigested *FLT3* D835 Master Mix Product	2.0 µL
HI-Deionized Formamide	4.0 µL
ROX size standards	1.5 µL
Blue Dextran loading dye	1.5 µL
Total Volume	**11.0 µL**

7.6.22. Prepare the samples containing **digested** *FLT3* D835 products by combining the reagents listed in Table 9 and mix well.

Table 9. Preparation of **digested** *FLT3* D835 products for detection

Reagent	Volume per Reaction
FLT3 ITD Master Mix Product	2.0 µL
digested *FLT3* D835 Master Mix Product	2.0 µL
HI-Deionized Formamide	4.0 µL
ROX size standards	0.5 µL
Blue Dextran loading dye	0.5 µL
Total Volume	**9.0 µL**

7.6.23. Prepare the samples containing template amplification control products by combining the reagents listed in Table 10 and mix well.

Table 10. Preparation of template amplification control products for detection

Reagent	Volume per Reaction
template amplification control product	2.0 µL
HI-Deionized Formamide	2.0 µL
ROX size standards	0.5 µL
Blue Dextran loading dye	0.5 µL
Total Volume	**5.0 µL**

7.6.24. Heat the reaction products to 94ºC for 2 minutes then snap chill on ice for 5 minutes.

7.6.25. Load the 5 µL of each preparation in separate wells of a preheated gel and run using the standard sequencing protocol.

7.7. Recommended Positive Controls

Table 11. Recommended Positive Controls

Master Mix	Target	Color	Control DNA	Cat#	Product Size (bp)
***FLT3* ITD**	*FLT3* Juxta-membrane Domain	**Blue & Green**	**Valid Size Range** IVS-0017 Clonal Control DNA	--- 4-088-0970	**300-1000**[1,2] 360
***FLT3* D835**	*FLT3* Kinase	**Blue**	**Valid Size Range** IVS-P001 Clonal Control DNA	--- --- 4-090-0010	**150 – undigested** **130 – digested** 188 – undigested 129 – digested
Specimen Control Size Ladder	Multiple Genes	**Blue**	**Valid Size Range** IVS-0000 Polyclonal Control DNA	--- 4-092-0010	**84, 96, 200, 300, 400, 600** 84, 96, 200, 300, 400, 600

Note: The amplicon sizes listed above were determined using an ABI 3100 platform. Amplicon sizes seen on your specific CE instrument may differ 1-4 bp from those listed above depending on the platform of detection (ABI) and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when tracking MRD.

[1]**Note:** To date, no activating *FLT3* deletions have been described in patients with AML.

[2]**Note:** Wild-Type and ITD peaks must be reported in both Green and Blue for a valid result. Peaks in a single color may be a result of cross-talk or nonspecific products.

8. Interpretation and Reporting

Although positive results are highly suggestive of malignancy, these assays are designed for Research Use Only and, if used in a clinical setting, can only be used in support of diagnosis. Interpret positive and negative results in the context of all clinical information and laboratory test results.

8.1. Sample Reporting

Results can be reported as "**Positive**" or "**Negative**" for "**Detection of mutations in the *FLT3* gene.**"

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

8.1.2. It is acceptable to call a sample "**Positive**" when a product is generated in the valid size range yet the positive control for that master mix fails.

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

8.1.4. All assay controls must be examined prior to interpretation of sample results.

The following describes the analysis of each of the controls and the decisions necessary based upon the results.

- **Negative Control**: (Polyclonal control, water or no template blank). If the negative control is:
  - **Positive**: Possible contamination of all PCR amplification reactions. Do not continue with the interpretation of results. Prepare fresh master mix and repeat amplification.
  - **Negative**: Continue with the analysis.
- **Positive Control**: (This can also be an extraction control if positive control material is taken through extraction processes). If the positive control is:
  - **Positive:** Continue with analysis.
  - **Negative:** Repeat assay unless specimen tests positive.
- **Specimen Control Size Ladder**: (This is run on unknown samples only). If the amplification control is:
  - **Positive**: ~100, 200, 300, 400, and 600 bp products are generated. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 bp fragment to have a diminished signal or to be missing entirely. Continue with analysis.
  - **Negative**: Repeat assay unless specimen tests positive.

8.2. Sample Interpretation

Following the acceptance of the controls, the samples are interpreted as follows:

8.2.1. *FLT3* ITD Master Mix:

- Positive: Presence of product(s) larger than 335 bp are reported as: "**Detection of internal tandem duplication mutation of the *FLT3* gene**."
- Negative: Presence of product(s) of approximately 331 bp are reported as: "**No evidence of an internal tandem duplication mutation of the *FLT3* gene**."

8.2.2. *FLT3* D835 Master Mix:

- **Positive:** Presence of product(s) of ~150 bp (undigested) and ~130 bp (EcoRV digested) are reported as: "**Detection of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene**."
- **Negative:** Presence of product(s) of ~150 bp (undigested) and ~81 bp (EcoRV digested) are reported as: "**No evidence of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene**."

9. Limitations of Procedure

The assay is subject to interference by degradation of DNA or inhibition of PCR due to heparin or other agents. The assay cannot reliably detect less than one positive cell per 100 normal cells.

10. Expected Results

The size range for each master mix has been determined testing positive control samples. For accurate and meaningful interpretation it is important to ignore peaks that occur outside of the valid size range for each master mix. Peaks that are outside of the range are not valid.

Note: "Color" indicates the color of products generated with the master mix when using differential fluorescence detection format (*e.g.*, ABI instruments).

10.1. Expected Size of Amplified Products

Table 12. Expected Size of Positive Controls

Master Mix	Target	Color	Control DNA	Catalog #	Product Size (bp)
***FLT3* ITD**	*FLT3* Juxta-membrane Domain	**Blue & Green**	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0017 Clonal Control DNA	--- 4-092-0010 4-088-0970	**300-1000**[1,2] 330 360
***FLT3* D835**	*FLT3* Kinase	**Blue**	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-P001 Clonal Control DNA	--- 4-092-0010 4-090-0010	**150 – undigested** 150 – undigested 80 – digested 188 – undigested 129 – digested
Specimen Control Size Ladder	Multiple Genes	**Blue**	Any Human DNA	---	84, 96, 200, 300, 400, 600

Note: The amplicon sizes listed above were determined using an ABI 3100 platform. Amplicon sizes seen on your specific CE instrument may differ 1-4 bp from those listed above depending on the platform of detection (ABI) and the version of the analysis software used. Once identified, the amplicon size as determined on your specific platform will be consistent from run to run. This reproducibility is extremely useful when tracking MRD.

[1]**Note:** To date, no activating *FLT3* deletions have been described in patients with AML.

[2]**Note:** Wild-Type and ITD peaks must be reported in both Green and Blue for a valid result. Peaks in a single color may be a result of cross-talk or nonspecific products.

10.2. Sample Data

Gel Detection: The data shown in Figure 2- Figure 4 was generated using the master mixes indicated.

- Lane 1 displays data generated testing the recommended 100% clonal control DNA.
- Lane 2 displays data generated testing a 10% dilution of the recommended clonal control DNA.
- Lane 3 displays data generated testing IVS-0000 Polyclonal Control DNA.

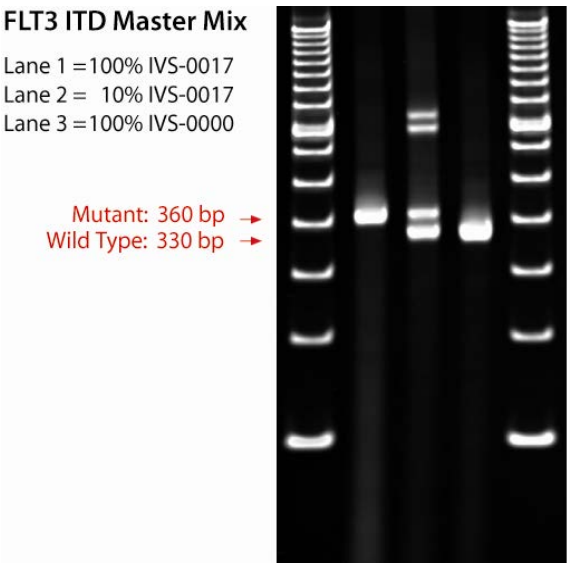

Figure 2. Amplified products were generated using the *FLT3* ITD master mix then run on a 2% agarose gel.

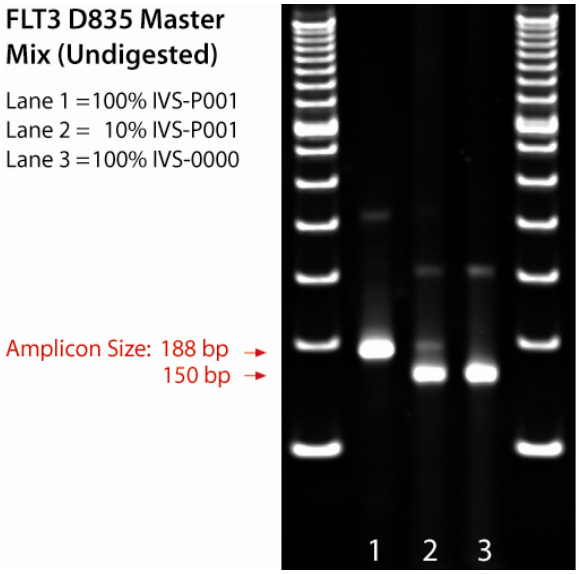

Figure 3. Amplified products were generated using the *FLT3* D835 master mix (undigested) then run on a 2% agarose gel.

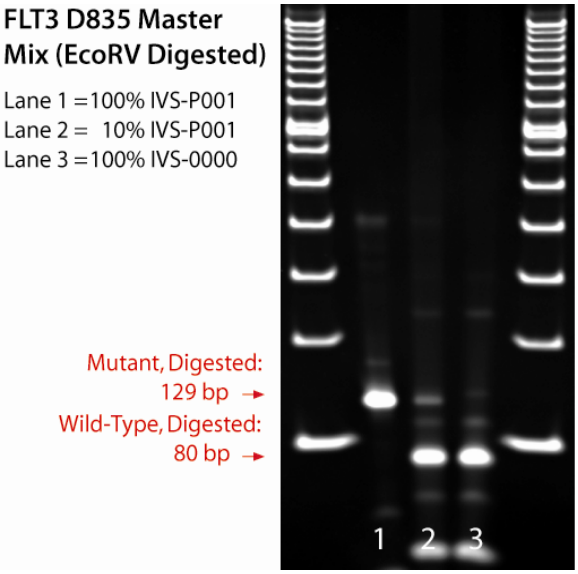

Figure 4. Amplified products were generated using the *FLT3* D835 master mix (EcoRV digested) then run on a 2% agarose gel.

ABI Fluorescence Detection: The data shown in Figure 5 and Figure 6 were generated using the master mixes indicated.

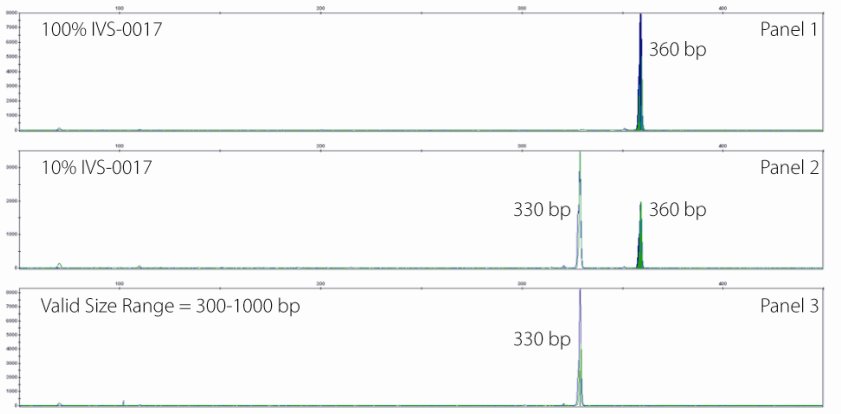

Figure 5. Amplified products were generated using the *FLT3* ITD master mix – 6FAM and HEX then run on an ABI 3100 instrument.

- Panel 1 displays data generated testing the recommended 100% clonal control DNA.
- Panel 2 displays data generated testing a 10% dilution of the recommended clonal control DNA.
- Panel 3 displays data generated testing IVS-0000 Polyclonal Control DNA

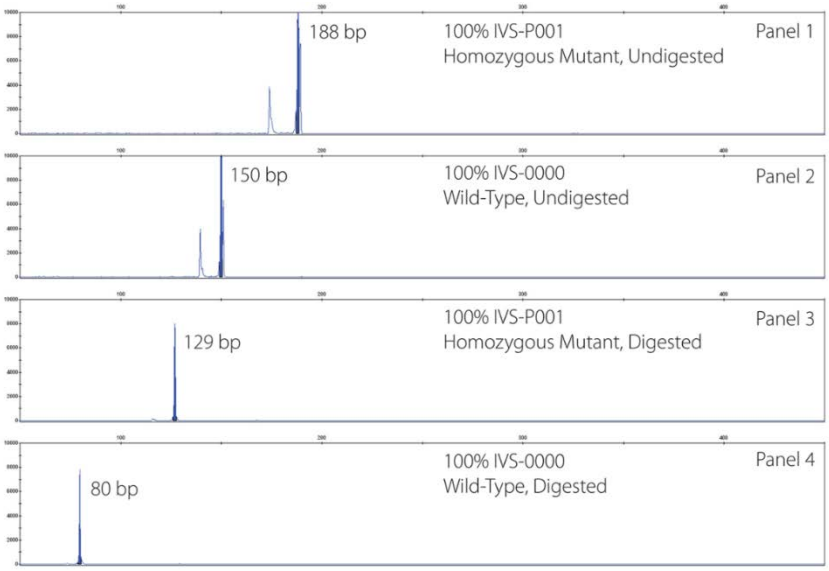

Figure 6. Amplified products were generated using the *FLT3* D835 master mix – 6FAM then run on an ABI 3100 instrument.

- Panel 1 displays data generated testing the undigested recommended 100% clonal control DNA.
- Panel 2 displays data generated testing the undigested IVS-0000 Polyclonal Control DNA.
- Panel 3 displays data generated testing the digested recommended 100% clonal control DNA.
- Panel 4 displays data generated testing the digested IVS-0000 Polyclonal Control DNA.

11. Technical and Customer Service

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

Contact Information

Invivoscribe, Inc
10222 Barnes Canyon Road, Bldg. 1
San Diego, California 92121-2711
USA

Phone:	+1 858 224-6600
Fax:	+1 858 224-6601
Technical Service:	support@invivoscribe.com
Customer Service	sales@invivoscribe.com
Website:	www.invivoscribe.com
Business Hours:	7:00AM – 5:00PM PST/PDT

Authorized Representative and EU Technical Assistance

EC REP Invivoscribe Technologies, SARL
Le Forum – Bât B
515 Avenue de la Tramontane
ZI Athélia IV
13600 La Ciotat, France

Phone:	+33 (0)4 42 01 78 10
Fax:	+33 (0)4 88 56 22 89
Technical Service:	support@invivoscribe.com
Customer Service	sales-eu@invivoscribe.com
Website:	www.invivoscribe.com
Business Hours:	9:00AM – 5:00PM CET/CEST

12. References

1. Levis M and Small D. (2003). *FLT3*: IT Does Matter in Leukemia. *Leukemia*. 17(**9**): p. 1738-52.
2. Murphy KM, Smith BD, Levis M, Small D and Berg KD. (2003). A Clinical PCR/Capillary Electrophoresis Assay for the Detection of Internal Tandem Duplication and Point Mutation of the *FLT3* Gene. *Journal of Molecular Diagnostics*. **5**:96-102.
3. Kottaridis, P.D., *et al*., (2001). The presence of a *FLT3* internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*. 98(**6**): p. 1752-9.
4. Levis, M, Tse, KF, Smith, BD., Garrett, E and Small, D. (2001). A *FLT3* tyrosine kinase inhibitor is selectively cytotoxic to acute myeloid leukemia blasts harboring *FLT3* internal tandem duplication mutations. *Blood*. **98**: 885-887
5. Yamamoto, Y., *et al.*, (2001). Activating mutation of D835 within the activation loop of *FLT3* in human hematologic malignancies. *Blood*. 97(**8**): p. 2434-9.
6. Rombouts, WJ, *et al.* (2000). Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the *FLT3* gene. *Leukemia*, 2000. 14(4): p. 675-83.
7. Kiyoi, H, *et al.*, (1999). Prognostic implication of *FLT3* and N-RAS gene mutations in acute myeloid leukemia. *Blood*. 93(**9**): p. 3074-80.
8. Kiyoi, H, Towatari, M, Yokota, S, Hamaguchi, M, Ohno, R., Saito, H. and Naoe, T (1998). Internal tandem duplication of the *FLT3* gene is a novel modality of elongation mutation that causes constitutive activation of the product. *Leukemia*. **12**: 1333-1337
9. Nakao, M, *et al.*, (1996). Internal tandem duplication of the *FLT3* gene found in acute myeloid leukemia. *Leukemia*. 10(**12**): p. 1911-8.

13. Symbols

The following symbols are used in Invivoscribe product labeling.

REF	Catalog Number		Expiration Date
VOL	Reagent Volume	EC REP	Authorized Representative in the European Community
LOT	Lot Number		Manufacturer
	Storage Conditions		Consult Instructions for Use

14. Legal Notice

This product is for Research Use Only; not for use in diagnostic procedures.

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

15. *FLT3* Mutation Assay: Single Page Guide

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

15.2. In a containment hood or dead air box, remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each master mix).

15.3. Aliquot volumes are 45 µL for each sample + 135 µL for the positive, negative and no template controls.

15.4. Add an additional 20 µL to correct for pipetting errors.

15.5. Add the appropriate amount of Taq polymerase (0.25 µL @5 U/µL per 50 µL total reaction volume) to each master mix and gently mix by inverting several times or gentle vortexing.

15.6. Aliquot 45 µL of master mix to individual wells of a PCR plate.

15.7. Add 5 µL of DNA from the unknown and control samples to individual tubes or wells containing the respective master mix reactions and pipet up and down several times to mix.

15.8. Amplify target DNA using the universal thermal cycler program.

Digest an aliquot of the *FLT3* D835 master mix products.

15.9. Add the components in the table below to a clean microfuge tube or individual wells of a 96 well plate.

Reagent	Volume per Reaction
FLT3 D835 Master Mix PCR reaction Products	10 µL
10X NEBuffer #3 (included with purchase of enzyme)	2 µL
EcoRV endonuclease (20 U/µL)	2 µL
Molecular Grade Water	16 µL
Total Volume	**30 µL**

15.10. Incubate at 37ºC for 60 minutes

Gel Detection – Agarose TBE Gels

15.11. Prepare a 2% MetaPhor or NuSieve 3:1 agarose/TBE gel.

15.12. Individually combine 20 µL from each amplification reaction with 4 µL of 6X gel loading buffer.

15.13. Load 20 µL of this mixture into separate wells of the gel, flanked by DNA size standards.

15.14. Detect products using ethidium bromide or an equivalent dye.

15.15. Photograph the gel and interpret the data.

ABI Fluorescence Detection with ABI 310 & 3100 instruments

15.16. Combine 1 µL of the *FLT3* ITD and undigested *FLT3* D835 Master Mix products in a tube and add 20 µL of HI-Deionized Formamide containing ROX size standards. Mix well.

15.17. Combine 1 µL of the *FLT3* ITD and **digested** *FLT3* D835 Master Mix products in a tube and add 20 µL of HI-Deionized Formamide containing ROX size standards. Mix well.

15.18. Add 1 µL of reaction product from the template amplification control in a tube and add 10µl of HI-Deionized Formamide containing ROX size standards. Mix well.

15.19. Heat reaction products to 95ºC for 2 minutes then snap chilled on ice for 5 minutes.

15.20. Prepare a **sample sheet** and **injection list** for the samples.

15.21. Review profile and controls, report results.