Instructions for Use

FLT3 Mutation Assay

RUO

For identification of internal tandem duplications and D835 mutations in the *FLT3* gene.

RUO This assay is 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 14120010	*FLT3* Mutation Assay for Gel Detection	33 Reactions
REF 14120031	*FLT3* Mutation Assay for ABI Fluorescence Detection	33 Reactions

Table of Contents

1. Assay Use

This Research Use Only assay identifies *FLT3* mutations.

2. Summary and Explanation of the Test

2.1. Background

FLT3 is a receptor tyrosine kinase that is normally expressed on many cell types including hematologic stem cells. Mutation of the *FLT3* receptor, by either internal tandem duplication (ITD) of the juxtamembrane domain or point mutation in the activation loop of the tyrosine kinase domain (TKD), causes constitutive activation of the *FLT3* receptor. Such gain-of-function mutations in the FMS related tyrosine kinase 3 (*FLT3*) gene are the subject of research studies and multiple clinical trials targeting Acute Myeloid Leukemia (AML) subjects.[1] The most prevalent type of *FLT3* mutation is an internal tandem duplication in and around the juxtamembrane domain.[2] The second most common mutation type in the *FLT3* gene is a TKD point mutation in aspartate (D835) or isoleucine (I836).

2.2. Summary

FLT3 Mutation Assays target 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. DNA is amplified by PCR, TKD amplicon is enzymatically digested, and *FLT3* mutations are detected via gel (REF 14120010) or capillary electrophoresis (REF 14120031).

Test kits include three PCR master mixes, along with positive and negative controls. *FLT3* ITD master mix tests for internal tandem duplication mutations. *FLT3* D835 master mix tests for TKD region mutations. The Specimen Control Size Ladder master mix targets multiple genes and generates a series of amplicons of approximately 100, 200, 300, 400, and 600 base pairs (bp) to ensure that the quality and quantity of input DNA is adequate to yield a valid result. Note that Specimen Control Size Ladder sizing was established using GeneScan™ 600 LIZ™ dye Size Standard v2.0 and that amplicon sizing can vary by up to 5 bp. Master mixes contain fluorophore-labeled (capillary) or unlabeled (gel) primer sets as appropriate to kit detection method.

3. Principles of the Procedure

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

FLT3 internal tandem duplication or length mutations are caused by duplication and insertion of a portion of the *FLT3* gene that includes the region in and around the juxtamembrane (JM) region of the *FLT3* gene. These mutations vary in both the location and the length of the inserted duplicated DNA sequence. ITD mutations result in constitutive autophosphorylation and activation of *FLT3*.[3] When interrogating human gDNA template on an ABI 3500xL genetic analyzer, wild-type *FLT3* alleles will amplify and produce a 327±1 bp product using this assay, while alleles that contain ITD mutations will produce a product that exceeds 327±1 bp (Figure 1).

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

FLT3 tyrosine kinase domain (TKD) mutations are caused by nucleic acid substitutions that result in a change in the amino acid sequence in this highly conserved catalytic center. TKD mutations, such as D835 and I836, result in constitutive autophosphorylation and activation of *FLT3*.[4] 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, rendering the EcoRV endonuclease unable to identify and digest the DNA at this site.

The *FLT3* target region is amplified using PCR and then an EcoRV restriction digest is performed. One of the PCR primers contains an EcoRV restriction site, so both wild type and mutant alleles are digested. The digestion pattern identifies loss of the normal gene sequence and ensures that digestion occurred. When interrogating human gDNA template on an ABI 3500xL genetic analyzer, wild-type alleles of the *FLT3* gene yield digestion products of 79±1 bp whereas mutant alleles yield products of 124+1 bp or 127±1 bp from the original undigested amplicon product of 145±1 bp or 147±1 bp, as measured by this assay (Figure 1).

3.3. Gel Detection

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

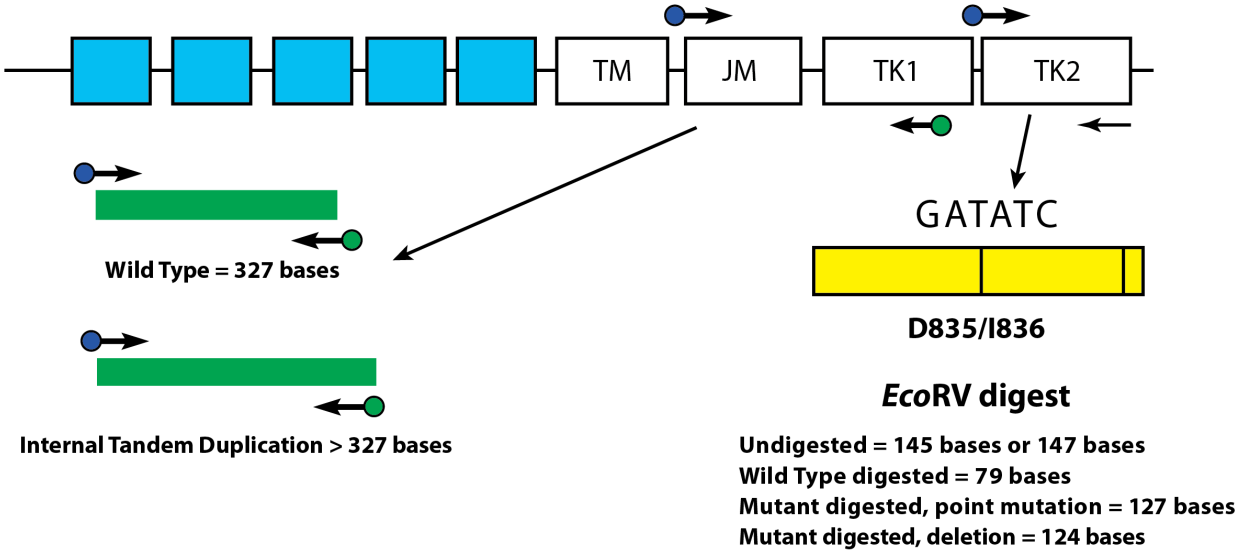


Figure 1. Depicted is a representation of the *FLT3* juxtamembrane (JM) region (TM = transmembrane) and the activating loop of the tyrosine kinase (TK) domain. Black arrows represent the relative positions of primers that target in and around the JM region for ITD or the activating loop of the kinase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

3.4. 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. Therefore, reaction products from several different master mixes can be pooled, fractionated using capillary electrophoresis and detected simultaneously. 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.

4. Reagents

4.1. Reagent Components

Table 1. Available Kits

Catalog #	Description	Total Reactions
REF 14120010	*FLT3* Mutation Assay for Gel Detection	33 Reactions
REF 14120031	*FLT3* Mutation Assay for ABI Fluorescence Detection	33 Reactions

Table 2. Reagent Components

Reagent	Catalog #	Reagent Components (active ingredients)	Unit Quantity	Assay Kit # of Units	Storage Temp.
Positive Control DNA	40880970	**IVS-0017 Clonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	2°C – 8°C or -85°C – -65°C
	40900010	**IVS-P001 Clonal Control DNA** 200 pg/mL of DNA in 1/10th TE solution	100 µL	1	
Negative Control DNA	40920010	**IVS-0000 Polyclonal Control DNA** 200 µg/mL of DNA in 1/10th TE solution	100 µL	1	
Gel Detection					
Master Mixes	24120010	***FLT3* ITD Master Mix - Unlabeled** Multiple oligonucleotides targeting the *FLT3* gene in a buffered salt solution.	1500 µL	1	-85°C – -65°C
	24120020	***FLT3* D835 Master Mix - Unlabeled** Multiple oligonucleotides targeting the *FLT3* TKD region in a buffered salt solution.	1500 µL	1	
	20960020	**Specimen Control Size Ladder - Unlabeled** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	
ABI Fluorescence Detection					
Master Mixes	24120011	***FLT3* ITD Master Mix – 6FAM & HEX** Multiple oligonucleotides targeting the *FLT3* gene in a buffered salt solution.	1500 µL	1	-85°C – -65°C
	24120031	***FLT3* D835 Master Mix – 6FAM** Multiple oligonucleotides targeting the *FLT3* TKD region in a buffered salt solution.	1500 µL	1	
	20960021	**Specimen Control Size Ladder – 6FAM** Multiple oligonucleotides targeting housekeeping genes.	1500 µL	1	

Note: There are no preservatives used during the manufacturing process of this kit.

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 assay kits at **-85ºC to -65ºC** until ready to use.
- Once kits are open, master mix storage at **-85ºC to -65ºC** is recommended. Store fluorophore-labeled master mixes in the dark.
- Once kits are open, DNA control storage at 2°C to 8°C or lower temperature is recommended.
- 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. Aliquot master mixes into sterile o-ring screw-cap tubes if necessary.

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
- 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 (gDNA). Common sources of gDNA include:

- 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA; OR
- Formalin-fixed paraffin embedded tissue or slides.

6.4. Sample Preparation

6.4.1. Using any method of DNA extraction, extract the gDNA from unknown samples.

6.4.2. 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.

- When testing unknown samples it is recommended to use a template amplification control master mix (*e.g.,* Specimen Control Size Ladder) to verify the absence of PCR inhibitors and ensure sufficient quality and quantity of DNA is present to yield a valid result.

6.5. Available Template Amplification Controls

6.5.1. The **Specimen Control Size Ladder** 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 ~100, 200, 300, 400, and 600 bp.
- 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 samples using a method that prevents degradation of DNA.

7. Assay Procedure

7.1. Materials Provided

Table 3. Materials Provided

Gel Detection Kits	
Catalog #	**Description**
REF 24120010	*FLT3* ITD Master Mix - Unlabeled
REF 24120020	*FLT3* D835 Master Mix - Unlabeled
REF 20960020	Specimen Control Size Ladder - Unlabeled
REF 40880970	IVS-0017 Clonal Control DNA
REF 40900010	IVS-P001 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

ABI Fluorescence Detection Kits	
Catalog #	**Description**
REF 24120011	*FLT3* ITD Master Mix – 6FAM & HEX
REF 24120031	*FLT3* D835 Master Mix – 6FAM
REF 20960021	Specimen Control Size Ladder – 6FAM
REF 40880970	IVS-0017 Clonal Control DNA
REF 40900010	IVS-P001 Clonal Control DNA
REF 40920010	IVS-0000 Polyclonal Control DNA

7.2. Materials Required (not provided)

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
DNA Polymerase	Roche: • EagleTaq DNA Polymerase or equivalent Invivoscribe: • FalconTaq DNA Polymerase or equivalent	05206944190 60970130	N/A
EcoRV Endonuclease Reagents	New England BioLabs: • EcoRV 4,000 U @20,000 U/mL	R0195S	NEBuffer™ r3.1 is included with purchase of EcoRV
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
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

Table 4. Materials Required (not provided)

Reagent/Material	Recommended Reagents/Materials and Suppliers	Part Number	Notes
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	Thermo Fisher Scientific: • Hi-Di™ Formamide	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: ○ GeneScan™ - 400HD [ROX]™	60980051 60980061 402985	N/A
Spectral Calibration Dye Sets	Thermo Fisher Scientific: • For ABI 3100 instruments: ○ DS-30 Matrix Standard Kit (Dye Set D) • For ABI 310 instruments: ○ And Fluorescent Amidite Matrix Standards [6FAM, TET, HEX, TAMRA, ROX]	4345827 401546	N/A
Polymer	Thermo Fisher Scientific: • POP-4™ Polymer: ○ POP-4 for 310 Genetic Analyzers ○ POP-4 for 3100/3100-Avant Genetic Analyzers	402838 4316355	N/A
Buffer	Thermo Fisher Scientific: • 10X Genetic Analyzer Buffer with EDTA	402824	Dilute 1:10 in sterile water before use

7.3. Quality Control

Positive and negative (or normal) controls are furnished with the kit and should be run in singlicate each time the assay is performed to ensure proper performance of the assay. In addition, include a no template control (*e.g.,* water) to test for contamination. The values for the positive controls are provided under section 10.1: *Expected Size of Amplified Products*. Amplicon sizes were determined using an ABI 3100 platform.

7.4. Reagent Preparation

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

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

7.4.2. In a containment hood or dead air box, remove an appropriate aliquot to 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.

7.4.3. 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 with subsequent addition of sample.

7.5. Amplification

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

7.5.2. 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

7.5.3. Amplify the reactions using the PCR program in Table 6:

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

Table 6. Thermal cycling conditions

Thermal Cycler Program			
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

7.5.4. Remove the amplification plate from the thermal cycler.

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

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

7.6.1. Prepare sufficient Digestion Master Mix for the number of samples to be digested.

- 20 uL Digestion Master Mix x number of samples to be digested

7.6.2. Prepare the NEB r3.1 restriction digest as detailed in Table 7.

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

Reagent	NEB r3.1 Restriction Digest
Molecular Grade Water (uL)	15.7 µL
10X NEBuffer™ r3.1 (uL)	2.3 µL
EcoRV endonuclease (20 U/µL)	2.0 µL
Digestion Master Mix (µL)	**20.0 µL**
FLT3 D835 Master Mix PCR amplicon	10.0 µL
Total Volume	**30.0 µL**

7.6.3. Aliquot 20 uL Digestion Master Mix per clean eppendorf tube or individual well of a 96-well plate.

7.6.4. Add 10 uL D835 amplicon sample to a 20 uL Digestion Master Mix Aliquot.

7.6.5. Seal plate or close tube(s).

7.6.6. Mix and digest samples at 37ºC for 60 minutes.

7.6.7. 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.

7.7. Detection

- Not all detection formats are available for all assays.

Gel Detection – Agarose TBE Gels (RECOMMENDED)

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

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

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

7.7.4. Detect products using ethidium bromide or an equivalent dye under UV illumination.

7.7.5. Photograph the gel and interpret the data.

Gel Detection – Polyacrylamide TBE Gels

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

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

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

7.7.9. 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.

7.7.10. Stain gels in 0.5 µg/mL ethidium bromide (in water or 0.5X TBE Buffer) for 5-10 minutes.

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

7.7.12. Visualize the gels with UV illumination.

7.7.13. Photograph the gel and interpret the data.

ABI Fluorescence Detection with ABI 310 & 3100 instruments

7.7.14. 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.

7.7.15. 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.

7.7.16. 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.

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

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

- As the samples are run on the machine, they are fractionated, detected, and analyzed by the instrument.
- A run is 20-24 minutes in duration.
- Data are automatically displayed as size and color specific peaks.

7.7.19. Review profile and controls, report results.

ABI Fluorescence Detection with ABI 373 & 377 instruments

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

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

7.7.21. 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 (diluted)	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**

7.7.22. 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 (diluted)	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**

7.7.23. Prepare the samples containing template amplification control (Specimen Control Size Ladder) 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 (diluted)	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**

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

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

8. Interpretation and Reporting

This assay is designed for Research Use Only; not intended for diagnostic purposes.

- Amplicon sizes were determined using an ABI 3100 platform. Amplicon sizes observed on each specific capillary electrophoresis (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 each specific platform will be consistent from run to run.
- The size range for each master mix has been determined by testing positive and negative control samples. For accurate and meaningful interpretation, ignore peaks (capillary detection method) that occur outside of the valid size range for each of the master mixes.
- Wild-Type and ITD peaks (capillary detection method) 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.

8.1. Reporting

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

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

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

8.2. Controls Interpretation

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.
- **Specimen Control Size Ladder** [Optional. This amplification control is recommended for samples of unknown quantity and quality.] ***If the Specimen Control Size Ladder:***
  - **Generates ~100, 200, 200, 400, and 600 bp products:** Continue with analysis. Note, as 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.
  - **Generates 1, 2, or 3 of the expected product sizes:** Re-evaluate sample for DNA degradation.
  - **Generates no products:** Repeat the assay and/or obtain a new specimen.

8.3. Samples

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

8.3.1. *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**."

8.3.2. *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.

10. Expected Results

10.1. Expected Size of Amplified Products

The amplicon sizes listed were determined using an ABI 3100 platform. Amplicon sizes observed on each specific capillary electrophoresis instrument may differ 1 to 4 base pair (bp) from those listed depending on the platform of detection and the version of the analysis software used. Once identified, the amplicon size as determined on each specific platform will be consistent from run to run.

Table 11. As a reference, the product sizes were determined using kit provided Master Mixes/Controls with capillary electrophoresis detection on an ABI 3100 platform.

Master Mix	Target	Color	Control DNA	Catalog #	Product Size (bp)
***FLT3* ITD**	*FLT3* ITD	**Blue & Green**	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-0017 Clonal Control DNA	--- 40920010 40880970	**300-1000[1]** 330 360
***FLT3* D835**	*FLT3* TKD	**Blue**	**Valid Size Range** IVS-0000 Polyclonal Control DNA IVS-P001 Clonal Control DNA	--- 40920010 40900010	**150 – undigested** **130 - digested** 150 – undigested 80 – digested 188 – undigested 129 – digested
Specimen Control Size Ladder	Multiple Genes	**Blue**	**Any Human DNA** IVS-0000 Polyclonal Control DNA	--- 40920010	**84, 96, 200, 300, 400, 600** 84, 96, 200, 300, 400, 600

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

[1]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

Data shown below was generated using kit provided master mixes and controls with product detection via 2% agarose gel or ABI 3100 capillary as indicated.

Gel Detection: The data shown in Figure 2 - Figure 4 was generated using the template plus master mix indicated with detection via a 2% agarose gel.

- Lane 1 data generated testing the recommended 100% clonal control DNA.
- Lane 2 data generated testing a 10% dilution of the recommended clonal control DNA.
- Lane 3 data generated testing IVS-0000 Polyclonal Control DNA, which is representative of a wild type product.

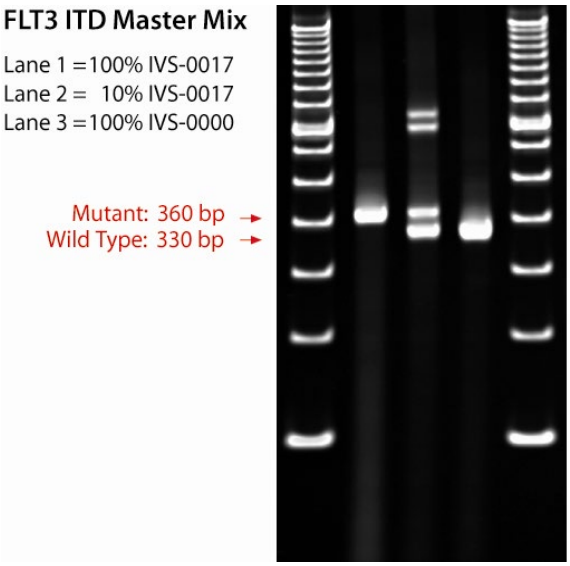


Figure 2. Amplified products were generated using the *FLT3* ITD master mix

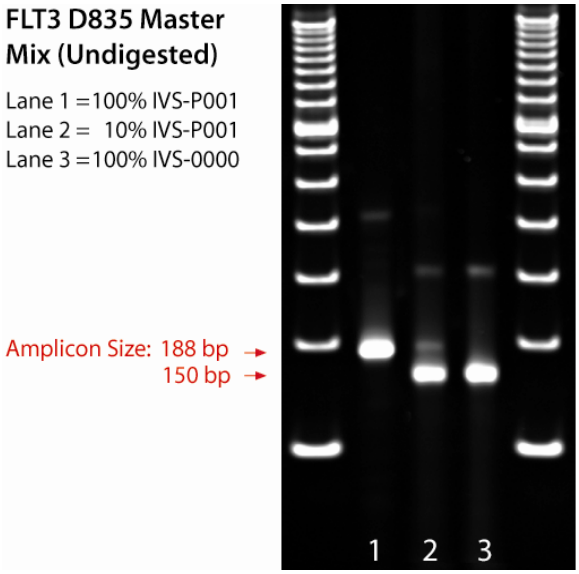


Figure 3. Amplified products were generated using the *FLT3* D835 master mix (undigested)

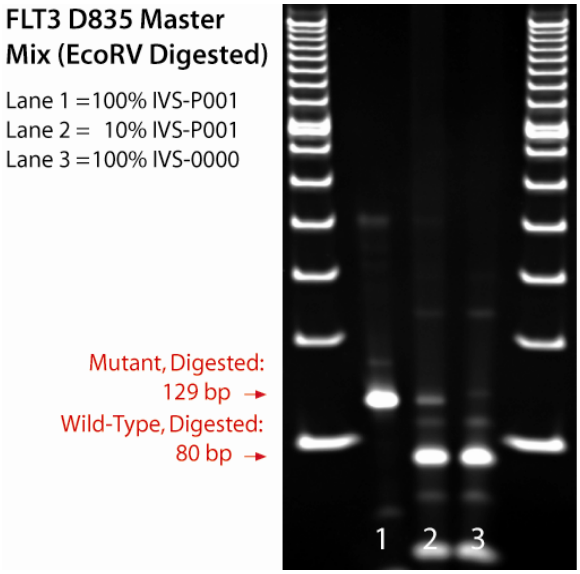


Figure 4. Amplified products were generated using the *FLT3* D835 master mix (EcoRV digested)

ABI Fluorescence Detection: The data shown in Figure 5 and Figure 6 were generated using kit provided master mixes and controls with detection on an ABI 3100 platform. *Note*: A 1-4 base pair (bp) difference in size detected is inherent to intra-instrument variance across capillary detection platforms.

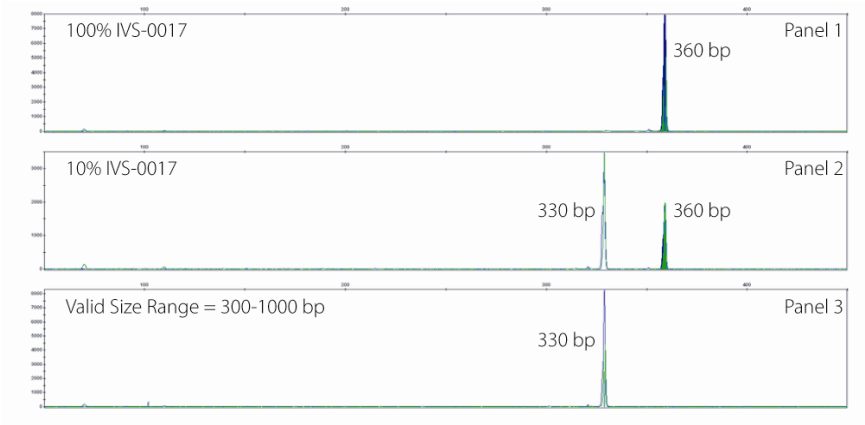
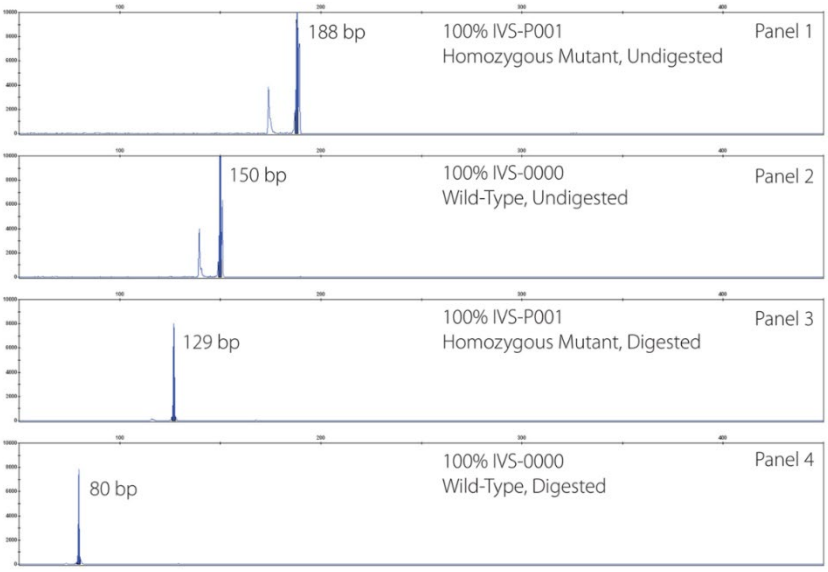


Figure 5 – FLT3 *ITD* Master Mix. Data panels demonstrate capillary peak detection of products amplified using the *FLT3* ITD master mix – 6FAM and HEX, and then run on an ABI 3100 instrument.

- Panel 1 displays data generated testing the recommended FLT3 ITD positive control.
- Panel 2 displays data generated testing a 10% dilution of the positive control.
- Panel 3 displays data generated testing IVS-0000 polyclonal control DNA, which is representative of a wild type product.

Figure 6 – FLT3 D835 Master Mix. Data panels demonstrate capillary peak detection of products amplified using the *FLT3* D835 master mix – 6FAM, and then run on an ABI 3100 instrument.

- Panel 1 displays data generated testing the undigested recommended 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 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.**

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

12. References

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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
RUO	For Research Use Only		

14. Legal Notice

14.1. Warranty and Liability

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

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

14.2. Patents and Trademarks

This product is covered by one or more of the following: European Patent Number 1549764, European Patent Number 2418287, European Patent Number 2460889, Japanese Patent Number 4708029, United States Patent 8859748, and related pending and future applications. All of these patents and applications are licensed exclusively to Invivoscribe®. Additional patents licensed to Invivoscribe covering some of these products apply elsewhere. 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.