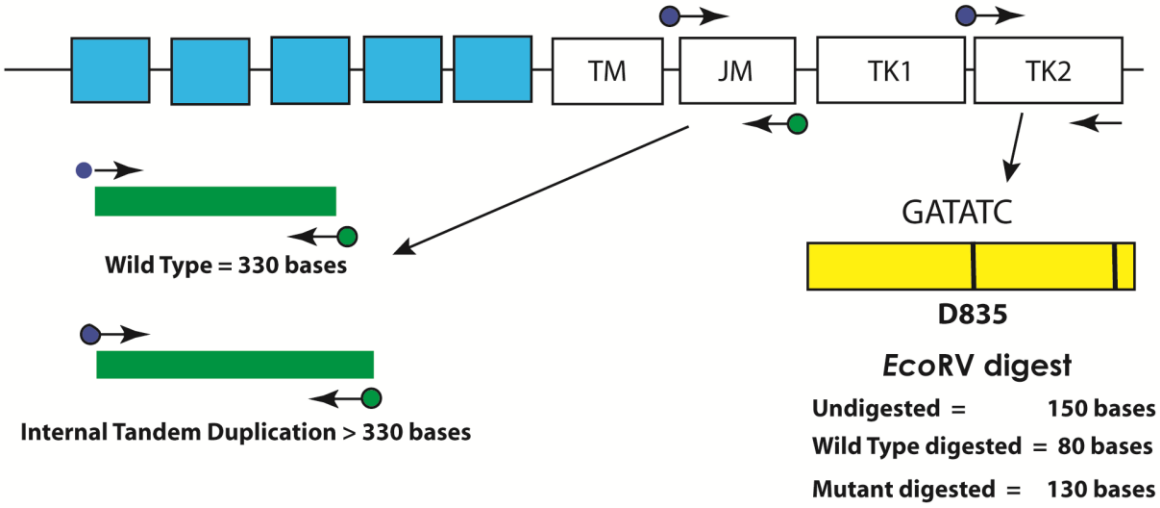
Instructions for Use

FLT3 Mutation Assay

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

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



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invivoscribe

Storage Conditions: **-65 ºC to -85 ºC**
(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

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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.** Questions received during business hours usually receive a response within an hour. Alternatively, you can call for technical assistance and for information on our testing kits at (858) 224-6600 between the hours of 7:00 AM and 5:00 PM PST/PDT.

1. Notice Regarding Patents

This product is covered by one or more of the following patents and patent applications owned by or exclusively licensed to Invivoscribe Technologies, Inc. (excluding the territory of Japan): United States Patent Numbers 6,846,630 and 8,178,292; European Patent Number 0959132 (validated in 15 EU countries); United States Patent Applications pending.

United States Patent number 6,846,630 and 8,178,292 (licensed exclusively to Invivoscribe Technologies, Inc. and owned by Takara Bio, Inc.) govern testing for the *FLT3* mutation within the United States**.** They also govern testing outside the United States when combined with transmission or importation of test results to the United States. Unless and except as expressly agreed in a writing signed by an officer of Invivoscribe, no license is conveyed by purchase and sale of this product for any such United States related product use where compensation for test results is charged or where test results are utilized for or in support of patient diagnosis or prognosis or added to patient files.

European Patent number 0959132 (licensed exclusively to Invivoscribe Technologies, Inc. and owned by Takara Bio, Inc.) governs testing (including research testing) for the *FLT3* mutation within Austria, Belgium, Denmark, Finland, France, Germany, Great Britain, Greece, Ireland, Italy, The Netherlands, Portugal, Spain, Sweden and Switzerland. The same patent governs testing (including research testing) elsewhere where importation of test results into any of these listed European countries for any use not covered by an express patent exemption is facilitated.

Included with the purchase price of this kit when sold to purchasers located within any of the European countries listed above is a limited license to perform no more than fifteen (15) tests requiring a European Patent license. Concurrent testing of positive, negative and blank controls do not count towards this total. Issuance of this limited license is contingent upon agreement to:

(i) maintain for three years (and make available on reasonable notice) records that reasonably demonstrate number of patent-covered tests performed; and

(ii) if results are published or transmitted outside the testing lab, include this notice language as part of the information delivery: "This test was performed pursuant to a license to European Patent 0959132, owned by Takara Bio Inc. of Otsu, Japan and licensed through Invivoscribe Technologies, Inc. of San Diego, USA."

For patent related reasons, this *FLT3* assay is not currently available for shipment to or use in, Japan.

Use of this product may require nucleic acid amplification methods such as Polymerase Chain Reaction (PCR). Any necessary license to practice amplification methods or to use amplification enzymes or equipment covered by third party patents is the responsibility of the user and no such license is granted by Invivoscribe Technologies, Inc., expressly or by implication. This product is sold **FOR RESEARCH USE ONLY; not for use in diagnostic procedures**.

For additional patent information, contact our legal department by email at legal@invivoscribe.com or by telephone in the United States: 1 (858) 224-6600.

2. 2. Principle

BACKGROUND:
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.

Gel electrophoresis is commonly used to resolve the different-sized amplicon products and ethidium bromide or other DNA intercalating dyes to stain and detect these products. A powerful alternative method is use of differential fluorescence detection with primers conjugated with fluorescent dyes that correspond to different targeted regions. 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 base resolution, differential product detection and relative quantification. In addition, the laboratory can eliminate the use of agarose and polyacrylamide gels, as well as the use of carcinogens such as ethidium bromide. Further, differential detection allows accurate, reproducible and objective interpretation of primer-specific products and automatic archiving of data. The limit of detection of this assay has been determined to be approximately 1 clonal cell in 100 hundred normal cells and inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis is approximately a single base pair. This reproducibility and sensitivity allows monitoring and tracking of individual tumors during research or methods development. The automatic archiving of specimen data allows comparison of data collected at different times.

This test kit includes 3 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 base pairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. A single thermal cycler program and similar detection methodologies are used with all of our standard assays. Many of our customers have remarked that this improves consistency and facilitates cross training on a broad range of different assays. These robust Invivoscribe assays can be used to test DNA extracted from virtually any source.

3. Assay Uses

***FLT3* Mutation Assays 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.

4. Specimen Requirements

- **This assay tests genomic DNA**

1. 5 cc of peripheral blood, bone marrow biopsy or bone marrow aspirate anti-coagulated with heparin or EDTA. Ship at ambient temperature; OR
2. Minimum 5 mm cube of tissue shipped frozen; or at room temperature or on ice in RPMI 1640; OR
3. 2 µg of genomic DNA; OR
4. Formalin-fixed paraffin embedded tissue or slides.

5. Kit Contents

Controls and Standards	**IVS Catalog #**	**Concentration**
IVS-0017 Clonal Control DNA	4-088-0970	100 µL @ 200µg/ml
IVS-P001 Clonal Control DNA	4-090-0010	100 µL @ 200pg/ml
IVS-0000 Polyclonal Control DNA	4-092-0010	100 µL @ 200µg/ml

Master Mixes for 1-412-0010 and 1-412-0020	**IVS Catalog #**	**Target**
FLT3 ITD Master Mix - Unlabeled	2-412-0010	*FLT3* juxtamembrane domain
FLT3 D835 Master Mix - Unlabeled	2-412-0020	*FLT3* kinase domain
Specimen Control Size Ladder - Unlabeled	2-096-0020	Multiple Genes

Master Mixes for 1-412-0031 and 1-412-0041	**IVS Catalog #**	**Target**
FLT3 ITD Master Mix - 6FAM & HEX	2-412-0011	*FLT3* juxtamembrane domain
FLT3 D835 Master Mix - 6FAM	2-412-0031	*FLT3* kinase domain
Specimen Control Size Ladder - 6FAM	2-096-0021	Multiple Genes

Note: MegaKits contain 10 units of each master mix and 5 units of each Controls and Standards

STATEMENT OF WARNINGS

The assay kit has been optimized to be used as a system. Do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit. Close adherence to the protocol will assure optimal performance and reproducibility. It is recommended that glass distilled de-ionized molecular biology grade water be used with the preparation of specimen DNA. This can be purchased from several manufacturers. In addition, laboratory personnel are reminded to wear appropriate personal protective equipment and follow good laboratory practices and universal precautions when working with specimens. Specimens should be handled in approved biological safety containment facilities and opened only in certified biological safety cabinets. Please see Section 9 for further details.

6. Storage Conditions

PCR master mixes are sensitive to freeze/thaw cycles. Therefore, for any duration other than immediate use, our **master mixes and assay kits should be stored at -65°C to -85°C**.

The reason for this is quite straightforward: Due to the high salt concentrations in our master mixes, the effective freezing and thawing temperature of the master mixes is approximately –10°C. The temperature in a standard laboratory –20°C freezer can easily reach –10°C or warmer during the day when these freezers are opened on a regular basis. At these temperatures, PCR master mixes may go through multiple freeze/thaw cycles, resulting in precipitation of the primers. Accordingly, to minimize the exposure of your master mixes to freeze/thaw cycles, **IVS recommends that master mixes be stored at -65°C to -85°C**.

Please note that our DNA standards are best stored at 2°C to 8°C. However, these standards can be stored at any lower temperature as long as they are vortexed after thawing and before use to ensure that they are re-suspended completely.

If you have any questions, please contact our technical staff. We are happy to help you determine your optimal storage needs.

7. Reagents Required But Not Included

PCR Amplification

AmpliTaq Gold DNA Polymerase or equivalent **(RECOMMENDED)**	(Life Technologies, Cat# N808-0241)
EagleTaq DNA Polymerase or equivalent **(RECOMMENDED)**	(Roche Cat# 05206944190)
AmpliTaq DNA Polymerase	(Life Technologies, Cat# N808-0161)
EcoRV restriction endonuclease	(New England Biolabs, Cat# R0195S)

ABI Fluorescence Detection

HI-DI Formamide with ROX size standards - ABI 310	(IVS, Cat# 6-098-0051)
HI-DI Formamide with ROX size standards - ABI 3100	(IVS, Cat# 6-098-0061)

8. 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.

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

Note[2] 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 aspecific products.

9. Procedure Notes

- Autoclaving does not eliminate DNA contamination.
- Work flow in the PCR laboratory should always be in a one way direction between separate work areas; beginning in Master Mix Preparation, moving to the Specimen Preparation, then to the Amplification and finally to Detection.

1. Do not bring amplified DNA into the areas designated for master mix or specimen preparation.
2. Due to the analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. All reagents should be closely monitored for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.
3. All pipettes, pipet tips and any equipment used in a particular area should be dedicated to and kept to that area of the laboratory.
4. PCR trays, bases and retainers must to be decontaminated in 10% bleach and rinsed with distilled water two separate times before returning them to the starting areas.
5. Sterile, disposable plastic ware should be used whenever possible to avoid RNase or cross-contamination.

10. Reagent Preparation

- All unknown samples should be tested using the template amplification control (**Amplification Control or Specimen Control Size ladder)** master mix. This is to ensure that no inhibitors of amplification are present and there is DNA of sufficient quality and quantity to generate a valid result.
- All samples should be tested in **singlicate**.
- **Positive**, **negative** and **no template** controls should be tested for each of the master mixes.

1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
2. In a containment hood or dead air box remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each of the master mixes). Aliquot volumes should be 45 µL for each sample + 135 µL (3 x 45 µL) for the positive, negative and no template (water) controls. We recommend adding an additional 20 µL to correct for pipetting errors.
3. Add the appropriate amount of AmpliTaq Gold or EagleTaq polymerase (0.25 µL of AmpliTaq Gold or EagleTaq @ 5U/µl per 50 µL total PCR reaction volume) to each of the master mixes 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.

11. Sample Preparation

Using any method of DNA extraction, extract the genomic DNA from unknown samples. Resuspend DNA to final concentration of 100µg - 400µg 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.

12. Amplification

1. Aliquot 45 µL of the master mix/enzyme solutions into individual PCR wells or tubes.
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, below. Pipet up and down several times to mix.

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

3. Amplify the reactions using the following PCR program:

(RECOMMENDED) **Standard Program for AmpliTaq Gold or EagleTaq**	Modified Program for AmpliTaq
Step 1: 95°C for **7 minutes**	Step 1: 95°C for **3 minutes**
Step 2: 94°C for 30 seconds	Step 2: 94°C for 30 seconds
Step 3: 55°C for 30 seconds	Step 3: 55°C for 30 seconds
Step 4: 72°C for 1 minute	Step 4: 72°C for 1 minute
Step 5: Go to step 2; 34 more times	Step 5: Go to step 2; 34 more times
Step 6: 72°C for 10 minutes	Step 6: 72°C for 10 minutes
Step 7: 4°C forever	Step 7: 4°C forever

4. Remove the amplification plate from the thermal cycler.

We recommend the MJ Research PTC-100, PTC-200 or the PE 2600, 9600 or 9700 thermal cyclers, using the following PCR parameters for the amplifications.

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

5. Digest the *FLT3* D835 Master Mix reaction products.
   Note: The restriction digest is NOT performed on the ITD master mix.

   Add the following to a clean microfuge tube or individual wells of a 96 well plate.

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

   Mix and place at 37ºC for 60 minutes.
   Place reactions on ice or refrigerate until ready for detection
   Note: If enzyme treatment is for less than the recommended 60 minutes, incomplete digestion may occur.

13. Detection

- **Not all detection formats are available for all assays**

Available Template Amplification Controls

- The **Amplification Control** master mix primers are labeled with a fluorescent dye (6-FAM). This label is detected as **BLUE** using the differential fluorescence software. The amplicons produced with this master mix are at 235 base pairs. The products of this master mix should be run separately.
- The **Specimen Control Size Ladder** master mix primers are labeled with a fluorescent dye (6-FAM). This 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 base pairs. 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. The products of this master mix should be run separately.

Gel Detection – Agarose TBE Gels (RECOMMENDED)

1. A 2% MetaPhor or NuSieve 3:1 agarose/TBE gel is prepared.
2. 20 µL from each of the amplification reactions are individually mixed with 4 µL of 6X gel loading buffer. 20 µL of this mixture is loaded into separate wells of the gel, flanked by DNA size standards. Products are detected using ethidium bromide or an equivalent dye.
3. Gel is photographed and data are interpreted.

Gel Detection – Polyacrylamide TBE Gels

1. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE, Invitrogen Cat# EC62652Box) and 0.5X TBE running buffer (Invitrogen 5X TBE Cat# LC6675).
2. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples.
3. Load 20 µL of mixture into wells of the gel.
4. 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.
5. Gels are stained in 0.5 µg/mL EtBr (in water or 0.5X TBE Buffer) for 5-10 minutes.
6. Gels are destained 2X in water for 5-10 minutes.
7. UV illumination is used for visualization.
8. Gel is photographed and data are interpreted.

Gel Detection – Heteroduplex Analysis

1. Denature 20 µL of PCR products at 94ºC for 5 minutes.
2. Re-anneal PCR products at 4ºC for 60 minutes.
3. Assemble electrophoresis unit using a 6% non-denaturing polyacrylamide TBE gel (made with 1X TBE, Invitrogen Cat# EC62652Box) and 0.5X TBE running buffer (Invitrogen 5X TBE Cat# LC6675).
4. Add 5 µL of ice-cold non-denaturing bromophenol blue loading buffer to samples
5. Load 20 µL of mixture into wells of the gel.
6. 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. Gels are stained in 0.5 µg/mL EtBr (in water or 0.5X TBE Buffer) for 5-10 minutes.
8. Gels are destained 2X in water for 5-10 minutes.
9. UV illumination is used for visualization.
10. Gel is photographed and data are interpreted.

ABI Fluorescence Detection with ABI 310 & 3100 instruments

1. 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.
2. 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.
3. 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.
4. Reaction products are heated to 95ºC for 2 minutes then snap chilled on ice for 5 minutes.
5. 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. Runs are 20-24 minutes in duration. The 310 & 3100 capillary electrophoresis instruments routinely handle 2 runs per hour (48 and 768 samples per day, respectively) and automatically analyze and store data for review or comparison with other test results.
6. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results.

ABI Fluorescence Detection with ABI 373 & 377 instruments

1. PCR Product Dilution: Initially dilute samples 1:10 in HI-Deionized Formamide or water (can be altered if the fluorescence signal is outside the optimal range).
2. Combine 2 µL of *FLT3* ITD and undigested *FLT3* D835 Master Mix products in a tube + 4 µL of HI-Deionized Formamide + 1.5 µL of ROX size standards + 1.5 µL of blue Dextran loading dye. Mix well.
3. Combine 2 µL of *FLT3* ITD and **digested** *FLT3* D835 Master Mix products in a tube + 4 µL of HI-Deionized Formamide containing + 0.5 µL of ROX size standards + 0.5 µL of blue Dextran loading dye. Mix well.
4. Add 2 µL of diluted reaction product from template amplification control in a tube + 2 µL of HI-Deionized Formamide containing + 0.5 µL ROX size standards + 0.5 µL blue Dextran loading dye. Mix well.
5. Reaction products are heated to 94ºC for 2 minutes then snap chilled on ice for 5 minutes.
6. Load the 5 µL of each of these preparations in separate wells of a preheated gel and run using the standard sequencing protocol.

14. Interpretation and Reporting

Note: This assay is for research use only. Although positive results are highly suggestive of malignancy, these assays are designed for Research Use Only and, if used in a clinical setting, should only be used in support of diagnosis. Positive and negative results should be interpreted in the context of all clinical information and laboratory test results.

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

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

Expected Size of Amplified Products

Master Mix	Target	Color	Control DNA	Cat#	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.

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

Note[2] 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 aspecific products.

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

1. Samples that fail to amplify following repeat testing should be reported as "**A result cannot be reported on this specimen because there was DNA of insufficient quantity or quality for analysis**".
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.
3. Samples that test negative should be repeated if the positive control reaction failed.
4. **All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples should not be interpreted**.

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

1. **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.
2. **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.
3. **Specimen Control Size Ladder**: (This is run on unknown samples only). If the amplification control is:
   - **Positive**: ~100, 200, 300, 400 and 600 base pair products are seen. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 base pair fragment to have a diminished signal or to be missing entirely. Continue with analysis.
   - **Negative**: Repeat assay unless specimen tests positive.

Sample Interpretation

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

1. ***FLT3* ITD Master Mix**:
   - **Positive:** Presence of product(s) larger than 335 base pairs are reported as: "**Detection of internal tandem duplication mutation of the *FLT3* gene**."
   - **Negative:** Presence of product(s) of approximately 331 base pairs are reported as: "**No evidence of an internal tandem duplication mutation of the *FLT3* gene**."
2. ***FLT3* D835 Master Mix**:
   - **Positive:** Presence of product(s) of ~150 base pairs (undigested) and ~130 base pairs (EcoRV digested) are reported as: "**Detection of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene**."
   - **Negative:** Presence of product(s) of ~150 base pairs (undigested) and ~81 base pairs (EcoRV digested) are reported as: "**No evidence of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene**."

15. 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 1 positive cell per 100 normal cells.

16. References

Levis M and Small D. *FLT3*: ITDoes Matter in Leukemia. Leukemia, 2003. 17(9): p. 1738-52.

Murphy KM, Smith BD, Levis M, Small D and Berg KD. A Clinical PCR/Capillary Electrophoresis Assay for the Detection of Internal Tandem Duplication and Point Mutation of the *FLT3* Gene. Journal of Molecular Diagnostics 2003, 5:96-102.

Kottaridis, P.D., et al., 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, 2001. 98(6): p. 1752-9.

Levis, M., Tse, K. F., Smith, B. D., Garrett, E. and Small, D: A *FLT3* tyrosine kinase inhibitor is selectively cytotoxic to acute myeloid leukemia blasts harboring *FLT3* internal tandem duplication mutations. Blood 2001, 98: 885-887

Yamamoto, Y., et al., Activating mutation of D835 within the activation loop of *FLT3* in human hematologic malignancies. Blood, 2001. 97(8): p. 2434-9.

Rombouts, W.J., et al., Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the *FLT3* gene. Leukemia, 2000. 14(4): p. 675-83.

Kiyoi, H., et al., Prognostic implication of *FLT3* and N-RAS gene mutations in acute myeloid leukemia. Blood, 1999. 93(9): p. 3074-80.

Kiyoi, H., Towatari, M., Yokota, S., Hamaguchi, M., Ohno, R., Saito, H. and Naoe, T: Internal tandem duplication of the *FLT3* gene is a novel modality of elongation mutation that causes constitutive activation of the product. Leukemia 1998, 12: 1333-1337

Nakao, M., et al., Internal tandem duplication of the *FLT3* gene found in acute myeloid leukemia. Leukemia, 1996. 10(12): p. 1911-8.

17. Appendix

Reagents and Special Supplies

Ficoll Separation

Ficoll-Hypaque or Ficoll-Paque	(Pharmacia, Cat# 17-0840-02)
1X PBS diluted from 10X PBS	(Gibco/BRL, Cat# 70011-044)
RPMI 1640	(Gibco/BRL, Cat# 11875-093)
DMSO Hybri-Max	(Sigma, Cat# D2650)
Fetal Bovine Serum	(Hyclone, Cat# SH30071.03)

Restriction Digestion

EcoR V (4,000 U @20 U/µL)	(NEB, Cat# R0195S)

Gel Electrophoresis

MetaPhor Agarose, 125 g	(Cambrex, Cat# 50180)
NuSieve 3:1 Agarose, 125 g	(Cambrex, Cat# 50090)
UltraPure™ 10 mg/mL Ethidium Bromide	(Invitrogen, Cat# 15585-011)
10X BlueJuice™ Gel Loading Buffer	(Invitrogen, Cat# 10816-015)
Ready-Load™ 100 bp Ladder	(Invitrogen, Cat# 10380-012)
Novex® TBE gels (6%, 12 well)	(Invitrogen, Cat# EC62652Box)
Novex® TBE Running Buffer (5X)	(Invitrogen, Cat# LC6675)
Novex® Hi-Density TBE Sample Buffer (5X)	(Invitrogen, Cat# LC6678)

Differential Fluorescence Detection

HI-DI Formamide with ROX size standards - ABI 310	(IVS, Cat# 6-098-0051)
HI-DI Formamide with ROX size standards - ABI 3100	(IVS, Cat# 6-098-0061)
HI-Deionized Formamide	(IVS, Cat# 6-098-0041)
HI-Deionized Formamide	(Applied Biosystems, Cat# 4311320)
GS ROX 50-400HD Size Standard	(Applied Biosystems, Cat# 402985)

18. Troubleshooting Guide

Our laboratories are located in San Diego, California. Technical assistance is most rapidly obtained using our Internet site: **http://www.invivoscribe.com** or by sending an email inquiry to: **support@invivoscribe.com**. Alternatively, you can call (858) 224-6600 for technical assistance and information on our testing kits between the hours of 7:00 AM and 5:00 PM PST/PDT.

Questions received during business hours usually receive a response within an hour.

19. Sample Data

Gel Detection

The data shown in Figures 1-3 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.

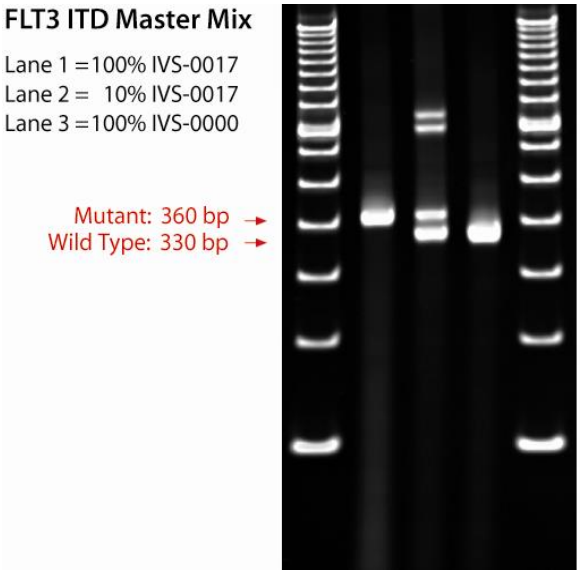

Figure 1. Amplified products were generated using the *FLT3* ITD master mix then run on a 2% agarose gel.

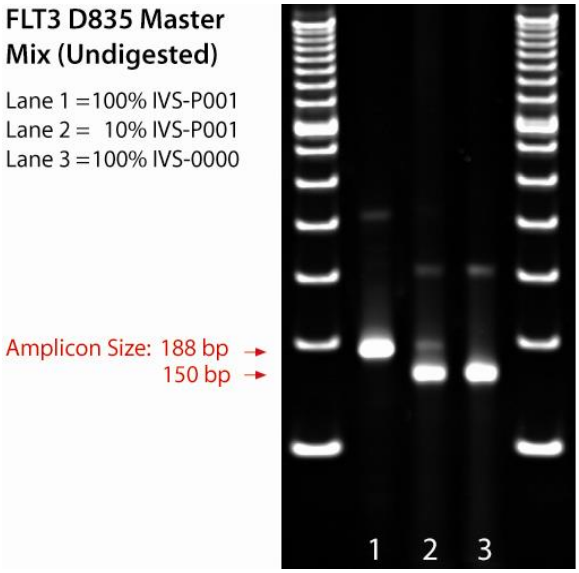

Figure 2. Amplified products were generated using the *FLT3* D835 master mix (undigested) then run on a 2% agarose gel.

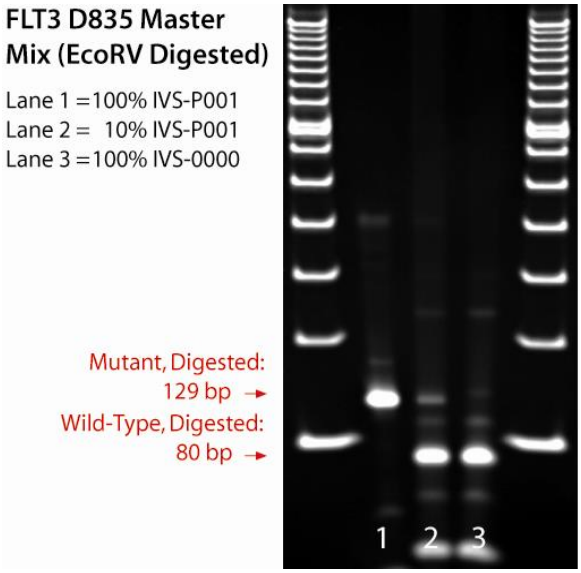

Figure 3. Amplified products were generated using the *FLT3* D835 master mix (EcoRV digested) then run on a 2% agarose gel.

ABI Fluorescence Detection

The data shown in Figures 4 and 5 were generated using the master mixes indicated.

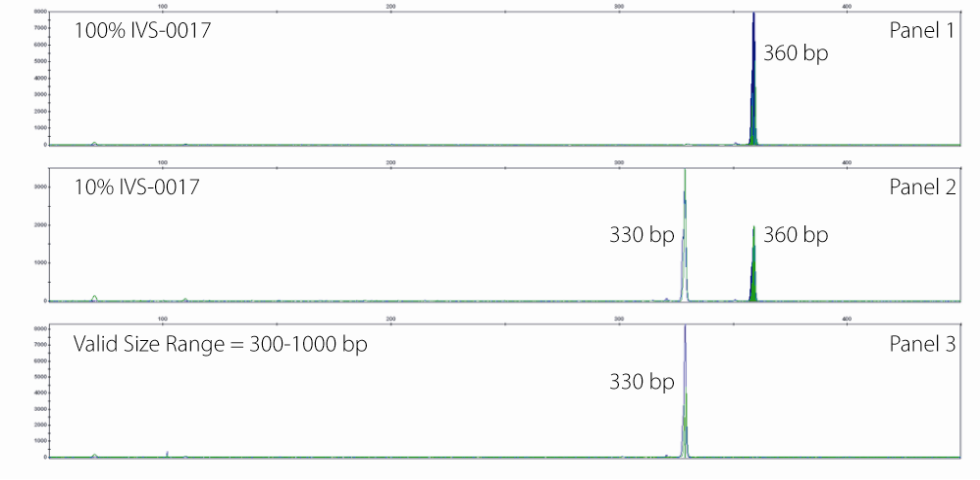

Figure 4. Amplified products were generated using the *FLT3* ITD master mix – 6FAM and HEX then run on an ABI 3100 instrument.

- 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

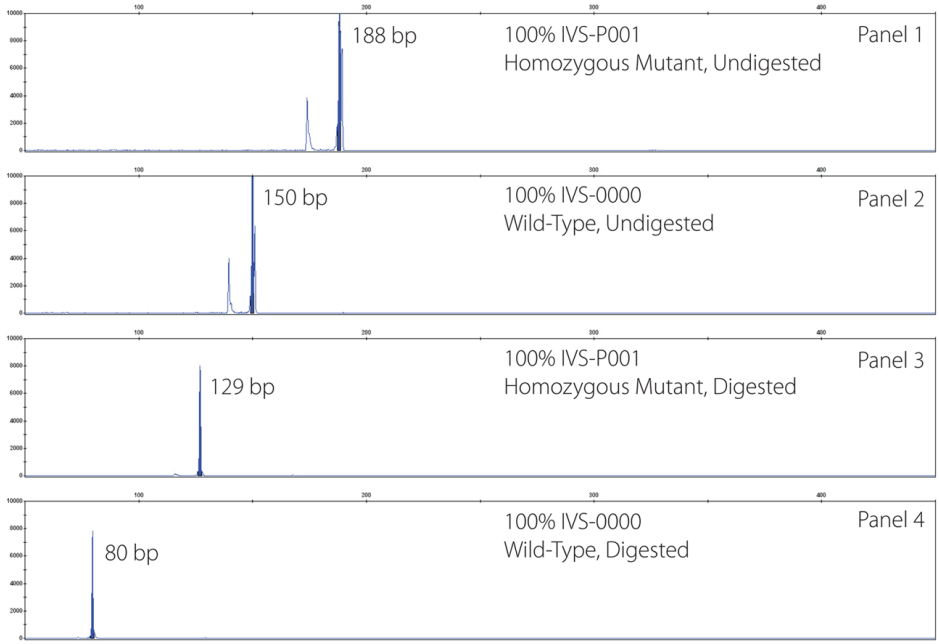

Figure 5. Amplified products were generated using the *FLT3* D835 master mix – 6FAM then run on an ABI 3100 instrument.

- 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.

20. *FLT3* Mutation Assay: Single Page Flow Chart

1. Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
2. In a containment hood or dead air box remove an appropriate aliquot to clean, sterile microfuge tube (one tube for each of the master mixes). Aliquot volumes should be 45 µL for each sample + 135 µL for the positive, negative and no template controls. We recommend adding an additional 20 µL to correct for pipetting errors.
3. Add the appropriate amount of AmpliTaq Gold or EagleTaq polymerase (0.25 µL of AmpliTaq Gold or EagleTaq @5 U/µL per 50 µL total reaction volume) to each of the master mixes and gently mix by inverting several times or gentle vortexing.
4. Aliquot 45 µL of master mix to individual wells of a PCR plate.
5. 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. Amplify target DNA using the universal thermal cycler program.
6. Digest an aliquot of the *FLT3* D835 master mix products.
   6.1. Add the following to a clean microfuge tube or individual wells of a 96 well plate.

   10 µL of the *FLT3* D835 Master Mix PCR Products

   2 µL of the 10X NEBuffer #3 (included with purchase of enzyme)

   2 µL of EcoRV endonuclease (20 U/µL)

   16 µL of Molecular Grade Water

   6.2. Incubate at 37ºC for 60 minutes

Gel Detection – Agarose TBE Gels

7. A 2% MetaPhor or NuSieve 3:1 agarose/TBE gel is prepared.
8. 20 µL from each of the amplification reactions are individually mixed with 4 µL of 6X gel loading buffer. 20 µL of this mixture is loaded into separate wells of the gel, flanked by DNA size standards. Products are detected using ethidium bromide or an equivalent dye.
9. Gel is photographed and data are interpreted.

ABI Fluorescence Detection with ABI 310 & 3100 instruments

7. 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.
8. 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.
9. 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.
10. Reaction products are heated to 95ºC for 2 minutes then snap chilled on ice for 5 minutes.
11. 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. Runs are 20-24 minutes in duration. The 310 & 3100 capillary electrophoresis instruments routinely handle 2 runs per hour (48 and 768 samples per day, respectively) and automatically analyze and store data for review or comparison with other test results.
12. Data are automatically displayed as size and color specific peaks. Review profile and controls, report results.