Instructions for use

Imegen® FLT3

Ref. IMG-238

CE IVD

Manufactured by:
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Code: HIC-PT-KIT 03-F-03 V.01

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Health in Code S.L. guarantees that all its products are free of defects, both in relation to the materials used and the manufacturing process. This guarantee is valid until the expiration date, provided that the storage conditions set out in this manual are followed.

Our products are designed for *in vitro* diagnostics. Health in Code S.L. makes no other express or implied guarantee, which extends beyond the proper operation of the components of this kit. The only obligation of Health in Code S.L. in relation to the aforementioned guarantees will be to replace the products or refund the purchase price thereof, as requested by the customer, provided that the defect in the materials or the manufacture of its products is proven. Health in Code S.L. shall not be liable for any direct or indirect damages resulting from economic losses or damages that may arise from the use of this product by the purchaser or user.

All the products marketed by Health in Code S.L. undergo rigorous quality control. Imegen® FLT3 has passed all internal validation tests, which guarantee the reliability and reproducibility of each manufactured batch.

For any questions about the applications of this product or the protocols thereof, please contact our Technical Department:

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Modifications to the instructions for use (IFU)		
Version 06	NOV 2022	Change of manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 05	SEP 2022	Change of manufacturer's identification: from Imegen to HEALTH IN CODE, S.L.
Version 04	JUL 2020	Updating the mutated/normal allele ratio from 0.78 to 0.5 in section 8 Analysis of results.

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01 General information

Acute myeloid leukemia (AML) is a hematologic malignancy of the myeloid lineage, characterized by the rapid proliferation of immature cells and the accumulation thereof in the bone marrow.

Constitutive activation of various members of the tyrosine kinase receptor family, including KIT, ABL, PDGFR and FLT3, is involved in the pathogenesis of this cancer. In the case of FLT3 (FMS-like tyrosine kinase-3, located on 13q12.2), activation frequently takes place by internal tandem duplications (ITDs) in the juxtamembrane domain, mainly in exon 14.

ITDs, which occur in 20-27% of AMLs, are variable in size but always a multiple of 3, so they do not alter the reading frame. The mutated/normal allele ratio has prognostic value, with a ratio greater than 0.5 being considered an indicator of increased risk of relapse.

References

- *Döhner, H. et al. Diagnosis and management of AML in Adults: 2017 ELN recommendations from an International Expert Panel. Blood [2017] 129, 424-447.*
- *Levis, M and Small D. FLT3: ITDoes matter in leukemia. Leukemia (2003) 17, 1738-1752.*
- *Thiede, C. et al. Analysis of FLT3-activating mutations in 713 patients with acute myelogenous leukemia (AML): High prevalence in FAB-subtype M5 and identification of subgroups with poor prognosis. Blood (2001) 98, 2994*

02 Intended use

Using the Imegen® FLT3 kit, ITDs in the *FLT3* gene can be analyzed by PCR and subsequent capillary electrophoresis.

PCR products will be separated by capillary electrophoresis, and will be detected by 6-Carboxyfluorescein (6-FAM) labeling.

Imegen® FLT3 is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

This kit has been validated using biobank samples, samples previously diagnosed by other laboratories with other technologies and synthetic plasmids made from the reference sequence containing the sequence with an ITD of 39 bp.

The material needed for this study is genomic DNA mainly from peripheral blood. The total quantity of DNA needed is 50 ng.

Following the protocol set out in Section 7 (Assay protocol), the limit of detection (LOD) has been set at 5%.

Health in Code S.L. is certified according to **the standard UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems – Requirements for regulatory purposes** by the AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS (AEMPS, Spanish Agency for Medicinal Products and Medical Devices) for the design, development and production of *in vitro* diagnostic medical devices:

- Genetic analysis kits
- Software for the bioinformatics analysis of genetic data

04 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not pipette by mouth.
- Do not smoke, eat, drink or apply cosmetics in the areas where kits and samples are handled.
- Any skin conditions, as well as cuts, abrasions and other skin lesions should be properly protected.
- Do not pour reagent residues into the drinking water system. It is recommended to use the waste containers set out by the legal regulations and to manage them via an authorized waste manager.
- In the case of accidental spillage of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with plenty of water.
- Material safety data sheets (MSDS) for all hazardous components contained in this kit are available upon request.
- This product requires the handling of samples and materials of human origin. It is recommended that all human-sourced materials be considered potentially infectious and handled in accordance with the OSHA Biosafety Level 2 standard for bloodborne pathogens or other relevant biosafety practices should be used for materials that contain or are suspected of containing infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive and do not cause biological environmental contamination.
- This kit has been validated with specific equipment and under specific conditions that may vary significantly in other laboratories. It is therefore recommended that each laboratory perform an internal validation when using the kit for the first time.
- The manufacturer is not responsible for the assay not working properly when the reagents included in the kit are replaced by other reagents not supplied by Health in Code S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user includes reagents not validated by Health in Code S.L., considering them equivalent to those supplied in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient reagents in order to make 33 determinations using the following reagents:

- ***FLT3 Master Mix:*** This is a PCR Master Mix with the specific oligonucleotides, dNTPs, $MgCl_2$ and buffer necessary to carry out amplification reactions.
- ***Taq:*** DNA polymerase necessary to carry out amplification reactions.
- ***Positive control:*** equimolar mixture of plasmid DNAs containing the normal sequence of exons 14 and 15 of the FLT3 gene, and the sequence with an ITD of 39 bp.

Reagents	Color	Quantity	Storage
FLT3 Master Mix	Blue pad	2 x 406 µL	-20°C
Taq	Yellow pad	25 µL	-20°C
Positive control	Blue cap	33 µL	-20°C

Table 1. Imegen® FLT3 kit components

06 Equipment, reagents and materials not included in the kit

Equipment:

- Thermal cycler
- 10 µL, 20 µL, 200 µL and 1000 µL micropipettes
- Vortex
- Centrifuge
- Capillary electrophoresis equipment

Reagents:

- *GeneScan™ 500 LIZ®* (Applied Biosystems cat. No. 4322682)
- *Hi-Di™ formamide*
- Nuclease-free water

Materials:

- Pipette tips with filter (10 µL, 20 µL, 200 µL and 1000 µL)
- 1.5 mL sterile tubes
- 0.2 mL tubes
- 96-well plates compatible with the capillary electrophoresis equipment
- Film for 96-well plates
- Latex gloves

NOTE: This kit does not include the reagents and materials necessary to perform capillary electrophoresis.

Complementary kits

For the diagnosis of acute myeloid leukemias, Health in Code S.L. also offers the real-time PCR kit Imegen® NPM1 **(ref. IMG-235).**

07 Assay protocol

07.1 | Preparation of amplification reactions

The Imegen® FLT3 kit is designed to perform one PCR reaction for each sample to be tested. In addition, it is recommended to include a negative control in each amplification batch to rule out the presence of contamination in the PCR reagents, and a positive control.

In order to estimate the quantity of reagents required, the number of samples and controls to be analyzed simultaneously must be taken into account. It is recommended to add one more reaction or increase the volume of each reagent by 10% when making the calculations.

The recommended protocol for the preparation of amplification reactions is shown below:

01 Thaw the *FLT3 Master Mix* tube, the positive control and sample DNA. Vortex each of the reagents and keep cold.

02 Prepare the reaction mix in a 1.5 mL tube by adding 22.5 µL of *FLT3 Master Mix* and 0.5 µL of Taq for each of the samples or controls. Vortex and spin.

03 Dispense 23 µL of the PCR mix in a 0.2 mL tube. Dispense as many 0.2 mL tubes as the number of samples or controls to be analyzed simultaneously.

04 In the corresponding 0.2 mL tubes, add 2 µL of the samples, previously diluted to a concentration of 25 ng/µL, 2 µL of nuclease-free water instead of DNA in the negative PCR control and 2 µL of the positive control.

05 Place the tubes in the thermal cycler and run the following amplification program:

◇ **Optimal program:**

Parameters	Stage 1	Stage 2			Stage 3	
No. of cycles	1 x cycle	30 x cycles			1 x cycle	
		Denaturation	Primer binding	Extension	Final extension and storage	
Temperature	94°C	94°C	60°C	72°C	72°C	4°C
Time	5 minutes	1 minute	1 minute	2 minutes	10 minutes	∞

Table 2. Optimal PCR program for Biometra T3 equipment, SimpliAmp Thermal Cycler and GENEAMP® PCR System 2720 (Applied Biosystems).

↘ It is possible to stop the protocol at this point. PCR products can be stored at 4°C if the protocol is to be continued within the next 24 hours or at -20°C for longer periods of time.

07.2 | Preparation of the fragment plate

In order to perform capillary electrophoresis, PCR products (fragments) must be prepared in a 96-well plate compatible with the capillary electrophoresis equipment as follows:

01 Add the following reagents to a 1.5 mL tube:

Reagents	Quantity per reaction
Formamide	18 µL
GeneScan™ 500 LIZ marker	0.5 µL

In the case of more than one reaction, increase the volume of each reagent by 10%.

NOTE: The volume of the size marker can be increased or decreased to adjust the intensity of the peaks observed in the electropherogram.

02 Dispense 18.5 µL of the above mixture into each well.

03 Add 1 µL of the DNA obtained from the PCR reactions.

NOTE: The sample volume can be increased or decreased (by diluting the samples with nuclease-free water) to adjust the intensity of the peaks observed in the electropherogram.

04 Seal the plate with a film, spin and denature in a thermal cycler for 5 minutes at 98°C.

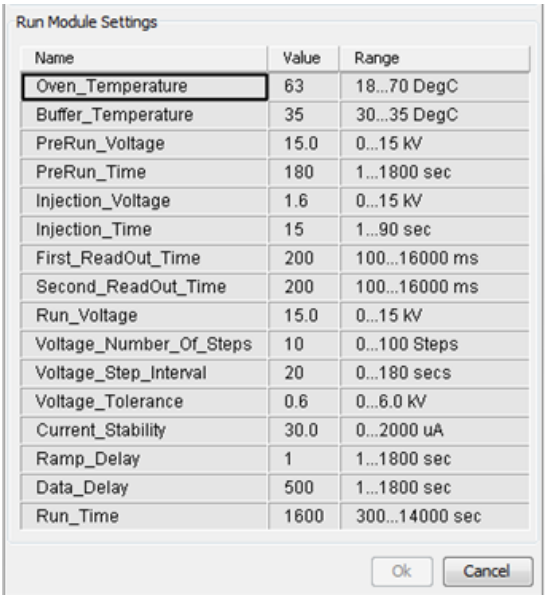
05 Store the plate at 4°C until it is put into the capillary electrophoresis equipment.

07.3 | Capillary electrophoresis

Once the fragment plate has been prepared, the reactions should undergo capillary electrophoresis. Depending on the sequencer model used, the electrophoresis conditions recommended by the manufacturer will be used.

In order to program such conditions, it should be considered that the amplification range varies approximately between 100 and 500 bp, that 6-FAM-labeled primers are used and that the molecular weight standard is labeled with *GeneScan™ 500 LIZ®*.

The following image shows the optimized conditions for the 3730xl DNA Analyzer sequencer (ThermoFisher Scientific), using the POP-7™ polymer.


Run Module Settings

Name	Value	Range
Oven_Temperature	63	18...70 DegC
Buffer_Temperature	35	30...35 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.6	0...15 kV
Injection_Time	15	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	15.0	0...15 kV
Voltage_Number_Of_Steps	10	0...100 Steps
Voltage_Step_Interval	20	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	1	1...1800 sec
Data_Delay	500	1...1800 sec
Run_Time	1600	300...14000 sec

Ok Cancel

Figure 1. Optimized parameters for the 3730xl DNA sequencer.

Detection intensity may vary between different equipment, depending on the model, the state of the optical system of the equipment, and the injection time and voltage. Therefore, it may be necessary to increase or decrease the quantity of size marker or PCR product required to perform capillary electrophoresis.

08 Analysis of results

The results are analyzed with a specific program for fragment analysis and the file obtained as a result of capillary electrophoresis. The result will be an electropherogram with peaks of a certain intensity (height) and a distance directly proportional to the size of the fragment (see Figure 2).

The size, in base pairs, of the fragment obtained indicates whether the sample analyzed has an expanded allele or not. In addition, the mutated allele/normal allele ratio can be calculated using the area of the peaks. A value over 0.5 is considered to carry a higher risk of patient relapse.

An image is shown below with examples of possible results.

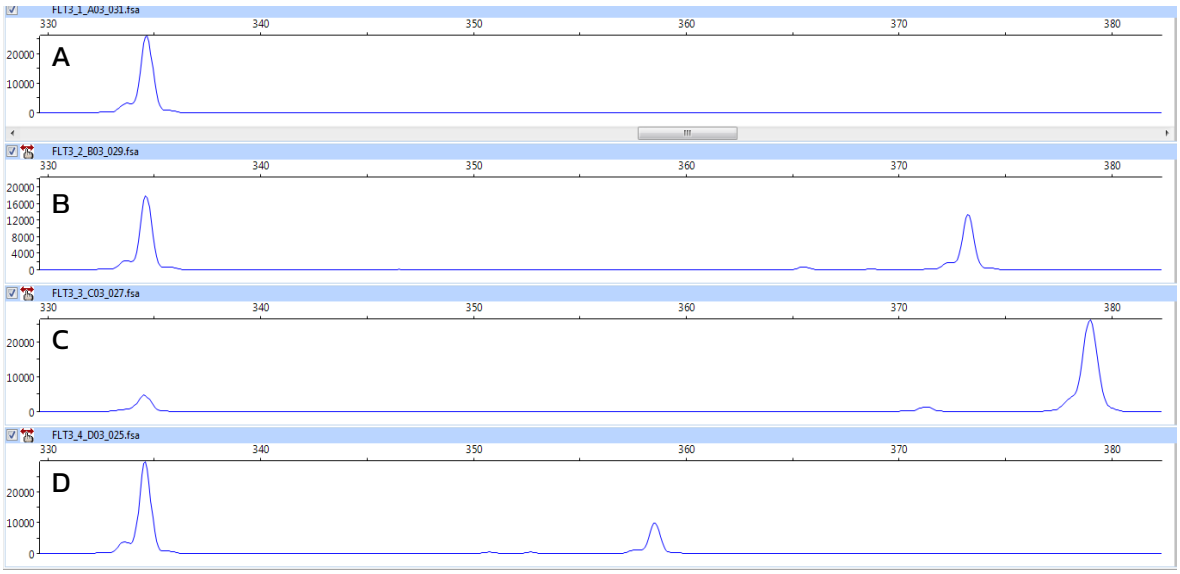


Figure 2. PCR results. Profile A of a DNA sample from a disease-free donor (335 bp). Profiles B, C and D of three DNA samples from AML patients with different FLT3-IDT mutations and thus three different sizes (insertion of 39, 45 and 24 bp, respectively); and various mutated/healthy ratios (0.74, 5.56 and 0.33).

An image of the result obtained for the positive control and its size in base pairs is shown below. The normal (unmutated) allele and an allele with the c.1785_1786ins39 mutation are present in this control.

Figure 3. Electropherogram obtained from the positive control

09 Troubleshooting

The following table shows the results that could be obtained for the analyzed samples, the positive control, the size marker and the negative control. In the case of an unexpected result, the interpretation and the most probable reason for such a result are given in the following table:

Problem	Samples analyzed	Positive control	Size marker	Negative control	Results/interpretation
Weak or no fluorescence signal				√	Expected result
	√			√	Insufficient quantity and/or quality of template DNA[1] Impure template DNA[2]
	√	√	√	√	Failed capillary electrophoresis[3] Failed denaturation[4]
	√	√		√	Failed PCR[5]
Excessive fluorescence signal	√				Excessive DNA quantity[6]
	√	√			
Presence of more peaks than expected		√		√	Contamination[7]

Table 3. Interpretation of possible results with the Imegen® FLT3 kit

(1) Insufficient quantity and/or quality of template DNA: Check that the DNA has been correctly quantified and use the indicated quantity of template DNA. If the DNA has been correctly quantified, check its integrity and perform a new extraction if necessary.

(2) Impure template DNA: High salt concentrations or altered pH can inhibit PCR. If you are using template DNA dissolved in an elution buffer with a pH other than 8 or at high EDTA concentrations, the volume of DNA should not exceed 20% of the total reaction volume. Traces of the reagents used during extraction can also affect the PCR reaction. If so, clean the DNA or prepare a new extraction.

(3) Failed capillary electrophoresis: Check if the equipment parameters are as specified by the manufacturer and reinject the samples.

(4) Failed denaturation: For a correct denaturation, the samples must be heated for the time indicated in section 7 of these instructions for use, and then kept cold until loading into the sequencer.

(5) Failed PCR: Check that the PCR program is the indicated one.

(6) Excessive DNA quantity: Make sure you are using the right quantity of DNA. If so, dilute the PCR product in sterile deionized water and prepare again for denaturation and loading into the sequencer.

(7) Contamination: This can be caused by another template DNA or by a previously amplified DNA. Cross-contamination can lead to false positives and negatives, resulting in problems in the interpretation of results. Use pipette tips with filters and change gloves regularly.

10 Limitations

10.1 | Equipment

Imegen® FLT3 has been validated using the following PCR thermal cyclers:

- *SimpliAmp Thermal Cycler* (ThermoFisher Scientific)
- *GeneAmp PCR System 2720* (ThermoFisher Scientific)
- *T3000 Thermocycler 48* (Biometra)

If you use another make or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical support for any questions or clarifications.

Imegen® FLT3 has been validated using the following sequencing platform:

- *3730xl DNA Analyzer* (ThermoFisher Scientific)

This kit is valid for polymers compatible with 6-Carboxyfluorescein (6-FAM) labeling. In the case of using equipment different from that mentioned above, follow the protocol specifications for those platforms.

10.2 | Reagents

Imegen® FLT3 has been validated using the reagents included in the kit and those recommended in section 6 of this document (Equipment, reagents and materials not included in the kit).

It is recommended to use the reagents recommended by the sequencer supplier for capillary electrophoresis: ThermoFisher Scientific.

In the case of doubt, please contact our technical support.

10.3 | Product stability

The optimum performance of this product is confirmed provided that the recommended storage conditions according to the optimum product date for each production batch are followed.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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