Instructions for use

FLT3-TKD2 dPCR OncoKit

Ref. IMG-405

RUO

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All Health in Code, S.L. products undergo strict quality control. The FLT3-TKD2 dPCR OncoKit product has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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01 General information

Acute myeloid leukemia (AML) is a neoplasm characterized by an abnormal proliferation of myeloid cells of clonal origin, which infiltrate the bone marrow, peripheral blood, and other tissues. Accumulation of these cells in different incomplete maturity stages due to failure to differentiate displaces healthy hematopoietic elements, leading to bone marrow failure and extramedullary infiltration in the spleen, liver, skin, gums, and central nervous system.

The immunological, cytogenetic, and molecular heterogeneity of adult acute myeloid leukemia patients below age 60 is related to the variability in their response to treatment and impacts global and disease-free survival. In Western countries, it accounts for 80% of acute leukemia cases in adults and 15-20% of cases in children under age 15, more commonly in newborns.

Mutations in the *FLT3* gene are strongly associated with a poor prognosis and a high count of leukemic cells in AML patients, suggesting that these are involved in disease progression. Between 15% and 35% of AML patients have been observed to carry mutations in this gene; therefore, their detection and analysis is relevant for diagnosis and follow-up.

The most common mutation is an internal tandem duplication (ITD) in the region encoding the juxtamembrane domain. The length of the duplication ranges from 3 to 400 bp, and it often occurs in exon 14, although it can occasionally include a portion of exon 15 as well.

Another mutation described in the *FLT3* gene is a point mutation in exon 20 of the second tyrosine kinase domain (TKD2). The most common one is p.D835Y, in which a G >T (GAT>TAT) substitution occurs in codon 835, leading to the replacement of aspartic acid by tyrosine. Other less common mutations exist, such as other deletions in codon 835 or deletions and insertions in codons close to 835, such as p.I836. Most patients carry only one type of mutation (ITD or p.D835), but both types have occasionally been found in the same patient.

The presence of FLT3-TKD2 mutations in AML patients treated with *FLT3* inhibitors (FLT3i) has been associated with the occurrence of resistance to treatment. Therefore, testing patients for these mutations could allow personalizing treatment with *FLT3* inhibitors, thus preventing the occurrence of resistance.

The following treatments approved by both the EMA *(European Medicines Agency)* and the AEMPS *(Spanish Agency for Medicines and Medical Devices)* are currently available in Spain: sorafenib, midostaurin, and gilteritinib.

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02 Intended use

FLT3-TKD2 dPCR OncoKit is designed to detect and quantify variants in codons 835 and 836 of the second tyrosine kinase domain of the *FLT3* gene from genomic DNA samples using digital PCR (dPCR) technology. The kit employs a combination of oligonucleotides and fluorescent hydrolysis probes in a non-competitive multiplex assay for quantification of the alternative allele.

FLT3-TKD2 dPCR OncoKit is intended for use in research and is aimed exclusively at healthcare workers and professionals working in molecular biology and in need of an accurate quantification of variants p.D835 and p.I836 in *FLT3*.

03 Technical characteristics

FLT3-TKD2 dPCR OncoKit is designed to analyze total genomic DNA (gDNA) extracted from peripheral blood samples.

To validate the kit FLT3-TKD2 dPCR OncoKit, independent synthetic DNAs were designed, containing both the mutated and the wild-type allele. The detection and quantification of both alleles allows calculating the frequency of the variant of interest (VAF, variant allele frequency) in the sample. Moreover, the reference sample FLT3 D835Y Reference Standard, 50% by Horizon Discovery (ID code: HD668), containing variant p.D835Y, has been used. Likewise, positive gDNA samples from patients that had been previously genotyped by massive sequencing (NGS) have been used.

The complete validation gives a robust and specific diagnosis method that allows establishing the following **technical specifications**:

- **Sample type**: Genomic DNA from peripheral blood
- **Positive control**: Synthetic DNA with heterozygous *FLT3* p.D835Y
- **Recommended amount of DNA**: 65 ng
- **Limit of detection**: 0.2% ± 5.4E-4
- **Limit of quantification**: 0.5%
- **Number of reactions per sample**: 1
- **Number of targets**: 2 (alternative allele and unchanged wild-type allele of *FLT3*).
- **Duration of the dPCR program**: 1 h 45 min.

FLT3-TKD2 dPCR OncoKit is compatible with the *QuantStudio™ Absolute Q™ Digital PCR System* (Thermo Fisher Scientific) platform, with FAM™ and VIC™ fluorescence channels. This device integrates the *QuantStudio Absolute Q Digital PCR Software CT* program for semiautomated result analysis.

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 mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory conduct an internal validation when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code, S.L.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code, S.L. but are considered by the user equivalent to those provided in the kit.

05 Content and storage conditions of the kit

FLT3-TKD2 dPCR OncoKit consists of the following reagents:

- **FLT3-TKD2 dPCR OncoKit Master Mix** containing:
  - specific oligonucleotides,
  - probes manufactured by Applied Biosystems™, FAM™- and VIC™-labeled to detect the wild-type and alternative alleles relative to variants in codons 835 and 836 of the TKD2 domain of the *FLT3* gene,
  - nuclease-free water.
- **Positive control**: contains synthetic DNA with variant p.D835Y in heterozygosity.

This kit contains enough freeze-dried reagents to carry out 24 digital PCR reactions. Table 1 summarizes the physical characteristics, amounts, and storage conditions.

Reagents	Color indicator	Quantity	Conservation
FLT3-TKD2 dPCR OncoKit Master Mix	Purple disk	2 X 12 rxn	4 °C
Positive control	Purple cap	6 rxn	4 °C

Table 1. Components of the kit FLT3-TKD2 dPCR OncoKit.

06 Equipment, reagents and materials not included in the kit

Equipment:

- 10 µL, 20 µL, and 200 µL micropipettes
- Vortex mixer
- Centrifuge
- *QuantStudio Absolute Q Digital PCR System* (Thermo Fisher Scientific)

Reagents:

- Nuclease-free water
- Absolute ethanol
- *Absolute Q DNA Digital PCR Master Mix* (Ref. A52490)
- *Isolation Buffer* (Ref. A52730)

Materials:

- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 ml tubes
- Powder-free latex gloves
- *MAP16 Plate Kit* (Ref. A52865)

Complementary kits

For the analysis of targets related to the study of hematologic pathologies, Health in Code, S.L. also offers the following real-time PCR kits:

- Imegen® BCR ABL1 Screening (ref. IMG-108)
- Imegen® m-BCR ABL (ref. IMG-121)
- Imegen® M-BCR ABL (ref. IMG-122)
- Imegen® Inv16 (ref. IMG-109)
- Imegen® PML-RARA Screening (ref. IMG-130)
- Imegen® PML-RARA (ref. IMG-111)
- Imegen® NPM1 (ref. IMG-235)
- Imegen® MPL (ref. IMG-236)

By fragment analysis:

- Imegen® FLT3 (ref. IMG-238)
- Imegen® CALR (ref. IMG-237)

By digital PCR analysis (dPCR):

- cKIT dPCR OncoKit (ref. IMG-407)

By massive sequencing (NGS, next-generation sequencing):

- Haematology OncoKitDx (ref. IMG-363)

07 Assay protocol

07.1 | Preparation of reagents for digital PCR

Before starting the protocol, the first step is to rehydrate freeze-dried reagents:

- ***FLT3-TKD2 dPCR OncoKit Master Mix***: add 20 µL of nuclease-free water to each vial.
- ***Positive Control:*** add 50 µL of nuclease-free water to each vial.

NOTE: storage at -20 °C is recommended once reagents are rehydrated.

07.2 | Preparation of amplification reactions

To estimate the amount of reagents needed, the number of samples and controls to be analyzed simultaneously must be taken into account. To perform the calculations, it is recommended either to add a sufficient amount of reagents to perform one extra reaction or to add an extra 10% of each reagent.

To carry out the quantitative analysis, we recommend performing one amplification reaction per sample and including the positive control, as well as a negative PCR control, to rule out any potential contamination of the reagents.

The protocol for the preparation of the amplification reactions is specified below:

01 Thaw all kit reagents, nuclease-free water, and DNA samples.

02 Shake and spin each reagent, then keep on ice.

03 Add the following reagents to a 1.5 mL tube:

Reagent	Volume per reaction
FLT3-TKD2 dPCR OncoKit Master Mix	1.5 µl
Absolute Q DNA Digital PCR Master Mix (5x)*	2 µL

(*) Reagent not provided by the manufacturer (see section "06. Equipment, reagents, and material not included in the kit").

04 Mix the PCR mix by pipetting up and down carefully to avoid bubbling and dispense 3.5 µL into the corresponding 0.2 mL tubes.

05 Add 6.5 µL of the DNA sample at 10 ng/µL or of nuclease-free water (negative PCR control) to the corresponding tubes.

06 At a 45° angle, load 9 µl of the PCR reagents onto the bottom of the well in the microfluidic array (MAP16) plate. Pipette the mix up to the first notch of the pipette.

07 Then, at a 45° angle, load 15 µl of Isolation Buffer against the side of the well and let it pour on the reagents to avoid mixing and bubbling.

07.3 | Settings for the digital PCR program

Loading the microfluidic array plate (MAP16)

Assemble the strips of the MAP17 plate into the four rows of wells. Make sure to completely cover the rows, then place the lids of the MAP plate on all the rows, including unused ones. Please download the *MAN0025621 QuantStudio™ Absolute Q™ Digital PCR System User Guide*, available at the www.thermofisher.com website, and follow the instructions in Section 2.

Settings for the PCR program

Place the MAP plate into the *QuantStudio™ Absolute Q™ System*, select the protocol from the list, and modify the optimal channels and parameters as specified in tables 2 and 3.

Fluorophore	Molecular target	Analysis
FAM™	codons 835 and 836 of *FLT3*	signal
VIC ™	endogenous *FLT3*	signal
ROX	QC	QC

Table 2. Channel settings for fluorophore detection in the QuantStudio™ Absolute Q™ dPCR System. QC: quality control.

Stage	No. of cycles	Temperature	Time
Initial denaturation	1	96 °C	10 minutes
PCR Denaturation, annealing, and extension	45	96 °C	10 seconds
		56 °C	15 seconds

Table 3. Optimal PCR program for the QuantStudio Absolute Q dPCR system (Thermo Fisher Scientific).

Array reading and generation of results

Once PCR is completed, please follow the instructions in the *Digital PCR QuantStudio™ Absolute Q™* User Guide, available at the www.thermofisher.com website.

08 Analysis of results

To analyze the results, it is important to know the labelling of the probes detecting each of the two targets analyzed by FLT3-TKD2 dPCR OncoKit. Table 4 shows the specifications for each one.

System	Fluorophore	Function	*Quencher*
FLT3-TKD2 dPCR OncoKit Master Mix	FAM™	The FAM™ probe is allele-specific for codons 835 and 836 of *FLT3*.	MGB
	VIC™	The VIC™ probe binds to a region of the non-mutated *FLT3* gene that is expected to be invariable. It is used as an endogenous control.	MGB

Table 4. Information about test probes. WT: wild-type.

The approach used by FLT3-TKD2 dPCR OncoKit is based on a non-competitive multiplex dPCR assay. Sample analysis is performed by the specific software for the PCR thermal cycler used: *QuantStudio™ Absolute Q™ Digital PCR Software*. This software allows displaying data in a two-dimensional (2D) scatterplot.

Setting the threshold for two targets in 2D plots allows identifying up to three clusters, or clouds (Figure 1):

- The cloud of black dots represents partitions where no amplification has taken place and, therefore, no signal has been detected for either probe.
- The cloud of orange dots corresponds to partitions that are only positive for the VIC™ probe, which hibridizes with an invariable region of the *FLT3* gene and which is used as an endogenous control.
- The cloud of green dots represents partitions where a signal has been recorded for both probes, FAM™ and VIC™ (combined cloud).

Due to the design strategy used, there is no possibility that a fourth cloud is generated in the upper left quadrant of the 2D plot. This is due to the fact that the FAM™ probe is designed to bind to the wild-type allele of the target codons. Simultaneously, the VIC™ probe is always amplified, as it hybridizes with a non-mutated region of the *FLT3* gene. Therefore, in the absence of a mutation, the highest possible FAM™ and VIC™ signals will be detected, since both of them bind to wild-type alleles, therefore only generating the green cloud, or combined cloud (Figure 1A).

Conversely, when the mutated allele is present, FAM™ will be decreased, and partitions will only appear when the endogenous *FLT3* control in VIC™, associated with the orange cloud (Figure 1 B-D), has been amplified. Table 5 summarizes the interpretations of the possible results.

Interpretation of results			
Sample	FAM™ cloud	VIC™ cloud	Combined cloud FAM™ + VIC™
Wild-type FLT3-TKD2	-	-	+
FLT3-TKD2 mutation	-	+	+

Table 5. Possible results when loading a gDNA sample, with + and – indicating the presence or absence of clouds or clusters.

Based on the generated plots, the analysis software reports the results as copies per microliter (cp/µL) according to the concentration of targets in the dPCR reaction. Based on these data, mutations in codons 835/836 (%) are quantified and expressed as a relative measure of the difference between the number of cp/µL detected for both wild-type alleles and the number of cp/µL of the *FLT3* gene, according to the following equation:

$$\%\ \boldsymbol{FLT3MUT} = \frac{(\boldsymbol{VIC} - \boldsymbol{FAM})}{\boldsymbol{VIC}}\ \boldsymbol{x}\ 100$$

Where:

- **FAM** is the number of copies/µL reported for FAM by the analysis software (allele region specific for codons 835-836 of the *FLT3* gene).
- **VIC** is the number of copies/µL reported for VIC by the analysis software (unaltered endogenous region of the *FLT3* gene).

For the run to be considered valid, the user must make sure that the following conditions are met:

- There is no amplification signal for the negative PCR control neither in the FAM™ nor in the VIC™ channels.
- A signal has been detected in both channels (FAM™ and VIC™) for the positive control reaction, with a quantification value of 50% (±10%).
- In case the obtained results differ from those described in this section, please see section "09. Troubleshooting" in this manual.

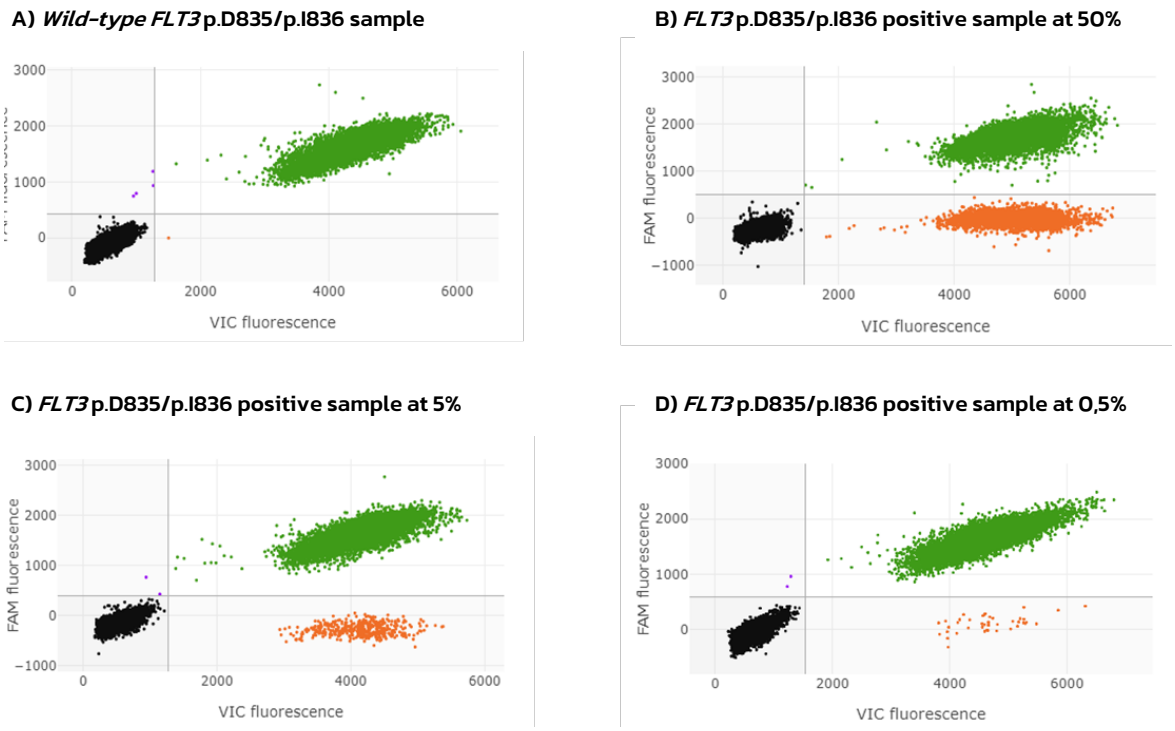

Figure 1. Two-dimensional (2D) plots obtained with the kit FLT3-TKD2 dPCR OncoKit, showing all the different result options. A) A green cloud is shown, as expected for a sample without mutations in FLT3. *The cloud corresponds to the combined FAM™ and VIC™ signals, amplifying both the invariable region of* FLT3 *and the wild-type allele of codons 835/836. B-D) Plots corresponding to positive samples for mutations in codons 835/836 of* FLT3. *As the frequency of the variant in the sample decreases, so does the orange cloud associated with the single amplification of the endogenous wild-type* FLT3 *region. In all the cases, the black cloud represents partitions where no amplification has occurred.*

09 Troubleshooting

Table 6 specifies the expected test results for the different controls and one sample in one run, along with their interpretation:

Control	2D scatterplot results			Cause
	FAM™	VIC™	FAM™ + VIC™	
Positive control	–	+	+	Expected result
	–	–	–	Failure of PCR amplification [1]
Sample	–	+	+	Expected result
	–	–	+	
	+	–	–	Invalid results [4]
	–	–	–	Failure of PCR amplification [2]
Negative PCR control	–	–	–	Expected result
	+	+	+	PCR contamination with human DNA [3]
	+	–	–	
	–	+	–	
	–	–	+	

Table 6. Interpretation of all possible results, with + and + indicating the presence or absence of clouds or clusters in the 2D plot.

(1) Failure of PCR amplification: make sure the amplification program and fluorescence detection settings are correct. An amplification error may be due to a technical issue during PCR program setup.

(2) Failure of sample amplification: verify that sample quantification meets the recommendations; if so, the specified result may be due to a highly degraded sample.

(3) PCR contamination by human DNA: PCR contamination may be due to mishandling of the sample, the use of contaminated reagents or environmental contamination. Thoroughly clean the laboratory where the PCR was prepared, as well as the equipment and material used. If necessary, use fresh aliquots of the PCR reagents. Prepare the PCR reaction containing the positive control last, in order to avoid cross-contamination. It is recommended that the assay be repeated in this case.

(4) Invalid result: no VIC amplification (endogenous control) is detected, and the run must therefore be repeated.

10 Limitations

10.1 | Analytical

FLT3-TKD2 dPCR OncoKit identifies and quantifies pathogenic variants in codons p.D835/p.I836 of the *FLT3* gene but cannot differentiate them. A different molecular technique needs to be used for that purpose.

FLT3-TKD2 dPCR OncoKit also detects variant R834Q, not associated with resistance to *FLT3* inhibitors.

10.2 | Equipment

FLT3-TKD2 dPCR OncoKit has been validated using the following amplification platforms:

- *QuantStudio™ Absolute Q™ Digital PCR System* (Thermo Fisher Scientific)

10.3 | Reagents

FLT3-TKD2 dPCR OncoKit has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment, reagents and materials not included in the kit).

10.4 | Product stability

Optimal performance of this product is achieved provided that the specified recommended storage conditions are applied, within the optimal product expiration date associated with each production batch.

11 Performance specifications

11.1 | Validation samples

FLT3-TKD2 dPCR OncoKit is designed to analyze total genomic DNA (gDNA) extracted from peripheral blood samples.

To validate FLT3-TKD2 dPCR OncoKit, different types of samples have been used:

- Independent synthetic DNA containing the alternative alleles p.R834Q/p.D835Y/p.M837I and wild-type.
- 23 clinical samples including 21 positive samples for variants in codon 835 (p.D835Y/H/E/A/N/V) and two positive samples for the most commonly recurrent change in codon 836 (p.I836del).
- The reference sample FLT3 D835Y Reference Standard, 50%, by Horizon Discovery (ID code: HD668).
- 16 negative samples for the target mutation.

11.2 | Limit of blank (LoB) and limit of detection (LoD)

The parameters limit of blank (LoB) and limit of detection (LoD) of FLT3-TKD2 dPCR OncoKit were determined using a *QuantStudio™ Absolute Q™ Digital PCR System*.

To calculate the LoB, 16 negative samples were used in two different runs. The expected negative result was obtained in all the cases, allowing to establish an LoB of 0.09±1.5E-4 % with a 95% CI. Taking into account the LoB obtained, the LoD was evaluated using reference samples diluted at different concentrations (1%, 0.5%, and 0.1%). Each dilution was loaded six times in the same run to ensure accurate results. The results obtained allowed establishing an LoD of 0.2±5,4E-4%.

11.3 | Limit of quantification (LoQ)

The limit of quantification of FLT3-TKD2 dPCR OncoKit was estimated on 6 replicates using reference samples diluted down to an allelic frequency of 0.5%. The results obtained met the criteria of acceptance to establish the limit of quantification at 0.5% (CV<25%).

11.4 | Specificity

The analytical specificity of FLT3-TKD2 dPCR OncoKit was determined by analyzing 16 negative samples for variants in codons 835 and 836.

In addition, the possibility of cross-reactivity with other variants in nearby codons was also assessed. For this purpose, synthetic DNA containing changes p.R834Q/p.M837I was designed. While no signal was detected for p.M837I, variant p.R834Q was detected. The latter variant is still relevant at the clinical level but is not associated with resistance to *FLT3* inhibitors.

11.5 | Repeatability and reproducibility

The repeatability and reproducibility parameters of FLT3-TKD2 dPCR OncoKit were estimated using a reference sample diluted down to the established LoQ (0.5%). This sample was loaded 8 times in a single run to estimate repeatability, reaching a value of 0.4±4,3E-4 with a 95% CI. For reproducibility, 4 replicates per run were performed in three different days (12 measurements in total). The reproducibility assays yielded a coefficient variation of 6.07%, which indicates an excellent accuracy for FLT3-TKD2 dPCR OncoKit. Tables 7 and 8 show the obtained results.

Replicate	Expected result (%)	Calculated result (%)
1	0.5%	0.39
2		0.36
3		0.45
4		0.44
5		0.32
6		0.43
7		0.31
8		0.48
Mean (%)		0.4
SD (%)		0.06
CI (95%)		0.4 ± 4.3E-4

Table 7. Results of the repeatability evaluation for FLT3-TKD2 dPCR OncoKit for samples assessed at the limit of quantification. SD: standard deviation. CI: confidence interval.

Assay	Expected frequency (%)	Relative frequency (%)	MEAN (%)	SD (%)	CV (%)
Day 1	0.5	0.34	0.48	0.03	6.07
		0.53			
		0.64			
		0.47			
Day 2		0.62	0.52		
		0.63			
		0.42			
		0.56			
Day 3		0.39	0.40		
		0.36			
		0.45			
		0.44			

Table 8. Results of the reproducibility evaluation for FLT3-TKD2 dPCR OncoKit for samples assessed at the limit of quantification. SD: standard deviation. CV: coefficient of variation.

11.6 | Sensitivity for clinical samples

The sensitivity of FLT3-TKD2 dPCR OncoKit was determined based on 23 clinical samples positive for mutations in the target codons (835 and 836), of which 21 has changes in codon 835 (p.D835Y/H/E/A/N/V) and two had changes in codon 836 (p.I836del). The allele frequency (VAF) for these variants ranged from 2% to 48.7%. The expected variant was correctly detected and quantified in all cases, therefore establishing a sensitivity of 100%.

Please contact our Technical Department if you have any questions about the use of this product or its protocols:

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