Instructions for use

Imegen® NPM1

Ref. IMG-235

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 its products are free of defects in both materials and workmanship. This guarantee remains in force until the expiration date, as long as the conservation practices described in this manual are adhered to.

Our products are intended for *in vitro* diagnostic use. Health in Code S.L. provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code's sole obligation, in relation to the aforementioned guarantees, shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship prove to be defective. Health in Code S.L. is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user as a result of use of the product by the buyer or user.

All Health in Code S.L. products undergo strict quality control. The Imegen® NPM1 kit 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, please contact our Technical Department:

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* **Imegen®** is a registered trademark in Spain of the Health in Code group

Instructions for Use (IFU) modifications		
Version 06	DEC 2022	Modification of the storage and shipping temperature of the GENERAL MASTER MIX reagent (Section 4).
Version 05	NOV 2022	Change in manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, España.
Version 04	SEP 2022	Change in manufacturer's identification: from Imegen to Health in Code S.L.
Version 03	JAN 2020	Correction in Section 1 (General information).
Version 02	JUN 2018	Document update for the product's CE-IVD.

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01 General information

Acute myeloid leukemia (AML) is a myeloid hematologic malignancy characterized by the rapid proliferation of immature cells that accumulate in the bone marrow.

Among the various mutational events involved in the pathogenesis of this cancer, mutations in the NPM1 gene, which codes for nucleophosmin, affect about 30% of AML patients, preferentially occurring in tumors without karyotypic abnormalities. The NPM1 gene, located at 5q35.1, encodes the *NPM1* protein, which is located in the nucleolus and involved in multiple functions, including regulation of the ARF-p53 tumor suppression pathway, centrosome duplication, and biogenesis and transport of ribosomes, among others. Mutations in *NPM1* (NM_002520.6) occur mostly in exon 11 in the form of a 4-nucleotide insertion that alters the reading pattern, with types A, B, and D being the most frequent mutations.

In the updated WHO classification, AML with mutated nucleophosmin (also known as cNPM+) constitutes a clinicopathological and molecular entity with clinical, risk stratification, prognostic, and therapeutic implications. Thus, the WHO established that the FLT3 gene should be studied simultaneously with *NPM1*, since mutations in the NPM1 gene have been associated with a better prognosis when they did not co-occur with other alterations, such as FLT3-ITDs.

References

- *Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. Leukemia. 2017;31: 798–807.*
- *Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006;107: 4011–4020.*
- *McClure RF, Ewalt MD, Crow J, Temple-Smolkin RL, Pullambhatla M, Sargent R, et al. Clinical Significance of DNA Variants in Chronic Myeloid Neoplasms. J Mol Diagnostics. 2018;20: 717–737.*

02 Intended use

The Imegen® NPM1 kit is aimed at the identification of mutations in the NPM1 gene through a qualitative real-time PCR assay that allows for the simultaneous amplification of mutations A, B, and D, located in exon 11 (NM_002520.6).

Wild-type	TATTCAAGATCTCTG----GCAGTGGAGGAAGTC
Variant A	TATTCAAGATCTCTCTCTGGCAGTGGAGGAAGTC
Variant B	TATTCAAGATCTCTGCATGGCAGTGGAGGAAGTC
Variant D	TATTCAAGATCTCTGCCTGGCAGTGGAGGAAGTC

This assay employs a combination of oligonucleotides and fluorescent hydrolysis probes in a validated analysis to detect the presence of both mutations in the NPM1 gene and the endogenous β-globin gene, used as an internal positive control for the genomic DNA sample. Testing for β-globin acts as an internal positive control of the DNA sample used in the assay.

The results obtained from this test can be used to confirm the patient's diagnosis. This test is not optimal for the study of minimal residual disease (MRD) in patients with AML.

Imegen® NPM1 can be used for *in vitro* diagnosis and is aimed at professionals working in molecular biology.

03 Technical characteristics

The Imegen® NPM1 kit has been validated with reference material obtained from the INCLIVA biobank. In addition, the validation included samples from diagnosed patients provided by the *Carlos Haya Regional University Hospital* (Málaga, Spain) and internal material from the *Medical Genetics Unit* of Health in Code S.L, as well as certified synthetic vectors (*GenScript*) containing the sequences of interest. This vector is included in the kit as the positive control, to verify the correct functioning of the PCR system. The complete validation gives a robust and specific diagnostic method.

The sensitivity and specificity of the *NPM1* detection system has been confirmed by in silico and empirical studies including wild-type samples and mutant samples previously genotyped using a different technology. The Limit of detection (LOD) has been set at 10%.

The type of sample required for this test is genomic DNA from peripheral blood. The total necessary amount of DNA for the assay is 50 ng.

Health in Code S.L. is certified under **UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems - Requirements for regulatory purposes** standard by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES (AEMPS) for the Design, development, and production of medical devices for *in vitro* diagnostic use:

- Genetic testing 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 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

The kit contains the following reagents necessary for the real-time PCR reactions, separated into the following components:

- **NPM1 Master Mix**: contains the oligonucleotides and probes for PCR amplification. FAM™-labeled probe for the specific detection of the amplified products of the A, B, and D forms of *NPM1*. VIC™-labeled probe for the specific detection of the amplified β-globin gene.
- **General Master Mix**: thermostable DNA polymerase mix, dNTPs, and other reagents necessary for PCR.
- **Positive Control**: positive control that contains sequence for amplification of type A *NPM1* mutation.

This kit contains enough reagents to perform 24 reactions.

Reagents	Color indicator	Quantity	Conservation
Master Mix NPM1	Yellow disc	180 µl	-20°C
General Master Mix	White cap	300 µl	-20°C*
Positive Control	Brown cap	25 µl	-20°C

Table 1. Components of the Imegen® NPM1 kit

(*) **General Master Mix**: Recommended to be kept frozen until first use, protected from light, and stored between 2- 8 °C after first use.

06 Equipment, reagents and material not included in the kit

Equipment:

- Real-time PCR thermal cycler (FAM and VIC channels)
- 10 µL, 20 µL, and 200 µL micropipettes
- Vortex mixer
- Centrifuge

Materials:

- Optical 96-well plates or 0.2 mL optical tubes
- Fungible optical material compatible with the real-time PCR thermal cycler
- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 mL tubes
- Latex gloves

Complementary kits

For the diagnosis of hematopoietic neoplasms, Health in Code S.L. also offers the following real-time PCR assays:

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

For the diagnosis of hematopoietic neoplasms, Health in Code S.L. also offers the following fragment analysis assays via capillary electrophoresis:

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

07 Assay protocol

07.1 | Preparation of amplification reactions

01 Thaw all kit reagents and RNA samples at room temperature and keep on ice once thawed.

02 Shake each reagent on a vortex mixer and keep cold.

03 In a 1.5 mL tube, add the following reagents to prepare the PCR mix:

Reagent	Volume per reaction
NPM1 Master Mix	7.5 µL
General Master Mix	12.5 µL

NOTE: To estimate the necessary amount of reagents according to the number of samples and controls that will be simultaneously analyzed in each run, we recommend either including one extra reaction in the calculations or increasing the volume of each reagent by 10%.

04 Mix on a vortex mixer, spin the PCR mix and dispense 20 µL into each well of the optical plate.

05 Once the PCR mixes have been dispensed, add the following amounts to the corresponding wells:

- 5 µL of genomic DNA samples (10 ng/µL).
- 5 µL of positive control
- 5 µL of nuclease-free water (negative control for PCR)

NOTE: It is recommended to add one negative PCR control per master mix to rule out reagent contamination, as well as one positive control per master mix to ensure the correct functioning of the PCR reaction.

06 Place the tubes or plates into the real-time PCR thermal cycler and configure settings for the amplification program as indicated in the next section.

07.2 | Settings for the real-time PCR program

- **Type of experiment**: Quantitation —Standard curve
- **Ramp rate**: Standard
- **Reaction volume**: 25 µL
- **ROX™ baseline reference**: included
- **Fluorophores of TaqMan® probes**:

Probe	Emitter	Genotype	Quencher
NPM1-P	FAM™	NPM1 alleles-A, B, and D	MGB
β-Globin-P	VIC™	β-Globin	MGB

Table 2. Information about hydrolysis probes

◇ Optimal program:

Fields	Phase 1 Enzymatic activation	Phase 2 PCR	
No. of cycles	1 initial cycle	40 cycles	
		Denaturation	Primer binding/extension
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	1 minute*

Table 3. Optimal PCR program for CFX96 (Bio-Rad), 7500 FAST, and StepOne Plus (Applied Biosystem)

(*) Fluorescence detection

08 Analysis of results

The following recommendations should be followed to ensure an adequate analysis of results:

- Make sure that no amplification occurred in negative PCR controls, either in the FAM or in the VIC channels. If amplification is detected, it is recommended to repeat the assay to rule out accidental contamination.
- Make sure that the positive control shows amplification of only *NPM1*, forms A, B, and D (FAM fluorochrome channel). No amplification of β-globin (VIC channel) shall be observed in the positive control.
- Make sure that amplification of the β-globin gene (VIC) occurred in all analyzed samples. No visible amplification of β-globin is an indicator of low-quality DNA from the sample and invalidates any further evaluation.
- Specific software must be used to analyze the samples. If a manual analysis is carried out according to the increase in fluorescence in each of the channels, the following observations shall be taken into account:

Below are the possible results obtained using the Imegen® NPM1 kit:

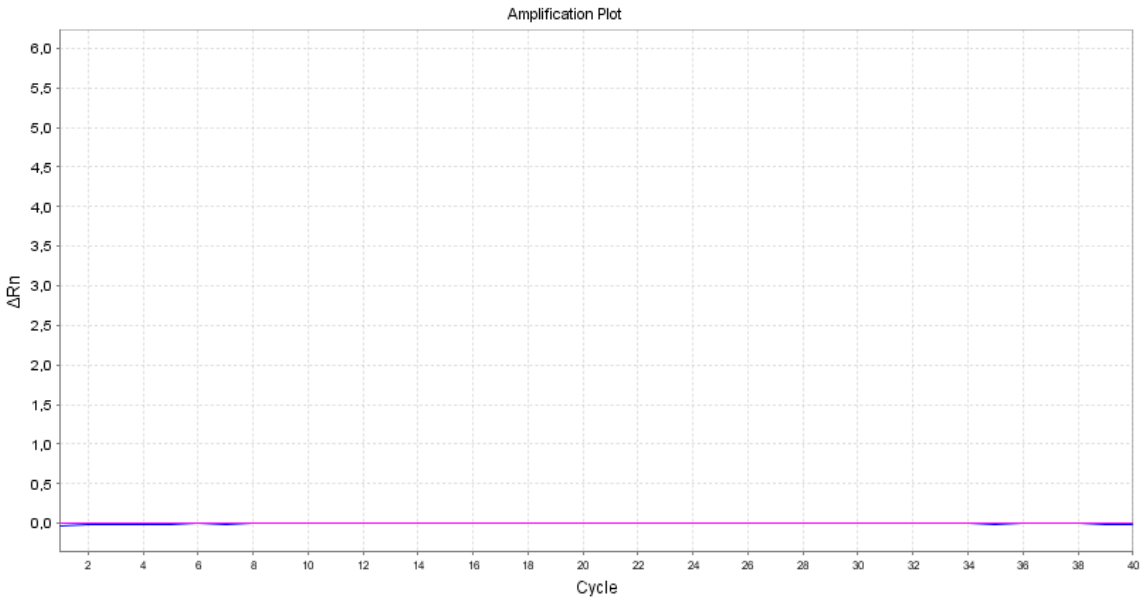

Figure 1. Expected result for the negative PCR control. No amplification signal is observed in any fluorescence channel.

gDNA SAMPLES

Verify that the reference gene is detected in all samples

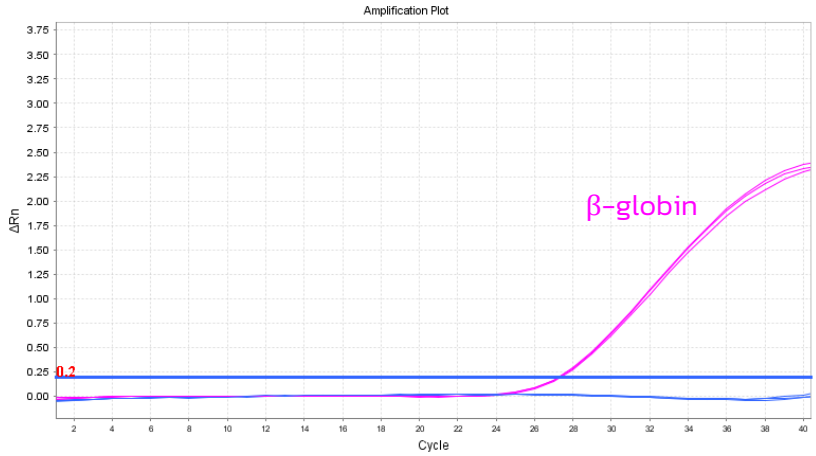

Figure 2. DNA sample without mutated NPM1 (or mutated forms other than A, B, or D): amplification will be observed only in the VIC channel (B-globin, shown in pink)

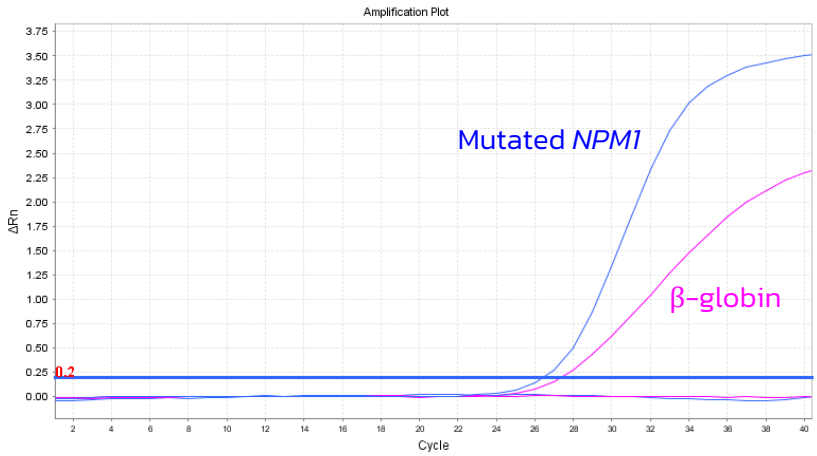

Figure 3. Expected result from a sample with mutated NPM1 (forms A, B, or D). Amplification is seen in both channels (in this plot β-globin is shown in pink and NPM1-A,B,D in blue)

POSITIVE CONTROL NPM1

Confirm that mutated NPM1 gene is detected

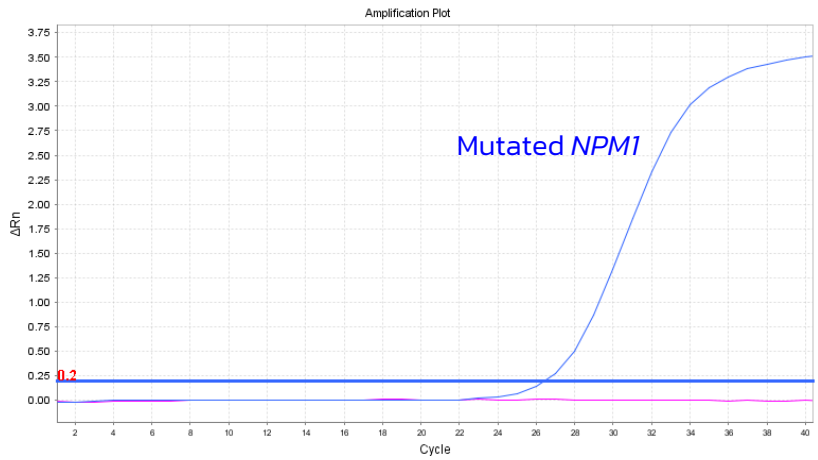

Figure 4. Expected result from the positive control. Amplification for mutated NPM1 is observed. NPM1-type A amplification is shown in blue

09 Troubleshooting

The following table shows results that may be obtained while using positive controls (Control NPM1), negative controls, and genomic DNA samples. If an unexpected result is obtained, the interpretation of the result and the most likely reason for the result are given in the following table:

Control	NPM1	β-Globin	Result / Interpretation
NPM1 control	+	–	Expected result
	–	–	Incorrect PCR settings [1]
DNA sample	–	+	Expected result
	+	+	
	–	–	DNA samples failed to amplify [2]
Negative control (NTC)	–	–	Expected result
	+	+	Contamination with human DNA or positive control material [3]

Table 5. Interpretation of the possible results from Imegen® NMP1

(1) Incorrect PCR settings: An amplification error may be due to a technical issue during PCR configuration. Make sure the amplification program and fluorescence detection configuration are correct.

(2) DNA sample failed to amplify: Failure to amplify the endogenous gene in the DNA sample might suggest that the quantity or quality of the DNA sample is compromised. In this case, it is advised to perform a new test or DNA extraction before a second attempt or interpreting the results.

(3) Contamination with human DNA or the positive control (Control NPM1): PCR contamination could be caused by improper sample handling, the use of contaminated reagents, or environmental contamination. To fix this issue, deep cleaning of the laboratory where PCRs are prepared is advised, including the equipment and material used. If necessary, use new aliquots from PCR reagents and finally prepare the PCR reactions containing the positive controls to avoid any cross-contamination.

10 Limitations

10.1 | Equipment

Imegen® NMP1 has been validated for use with the following PCR thermal cyclers:

- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Real-Time PCR System* (ThermoFisher Scientific)
- *CFX96 Real-time PCR System* (BioRad)

If a different brand or model of thermal cycler is used, the amplification program may need to be adjusted. Should you need further information or advice, please contact our technical support service.

10.2 | Reagents

Imegen® NMP1 has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment and materials not included in the kit).

10.3 | 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.

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

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