Instructions for use

Imegen® CALR

Ref. IMG-237

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 from defects, both in the used materials and in its manufacturing process. This warranty is extended to the expiration date, as long as the storage conditions specified in this manual are met.

Our products are designed for *in vitro* diagnostic use. The user of the product is responsible for validating the usefulness of the protocol proposed by Health in Code S.L.. Health in Code S.L. does not offer any other warranty, express or implied, which extend beyond the proper functioning of the components of this kit. Health in Code S.L. sole obligation in respect of the preceding guarantees, will be to replace the product or return the purchase price thereof, as desired by the customer, as long as the existence of a defect in the materials or in the manufacture of its products is identified. Health in Code S.L. will not be responsible for any damage, direct or indirect, resulting in economic losses or damages resulting from the use of this product by the purchaser or user.

All products sold by Health in Code S.L are subjected to rigorous quality control. The Imegen® CALR kit has passed all internal validation tests, ensuring 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 05	NOV 2022	Change of the manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 04	SEP 2022	Change of the manufacturer's identification, going from Imegen to Health in Code S.L.
Version 03	JUN 2018	Change implemented: adaptation to the requirements of Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017, on *in vitro* diagnostic medical devices.

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01 General information

Myeloproliferative neoplasms (MPN) are a type of chronic myeloid cell cancer where an overproduction of precursors to blood cells in an advanced state of maturation. In two subtypes of these neoplasms, essential thrombocythemia and primary myelofibrosis, the calreticulin gene (CALR) (NM_004343.3) is mutated in around 70-88% of those cases not affected by mutations in the JAK2 gene, which is the main oncogenic event among this type of neoplasms.

The CALR gene is a highly conserved gene that encodes a protein found in a wide range of subcellular localizations and with the ability to bind Ca2+ cations. Its mutation consists of insertions and deletions in exon 9, which alter the reading frame and lead to an alternative C-terminal end of the protein. These mutations have been associated with two hematologic disorders: essential thrombocythemia and primary myelofibrosis.

References

- *Klampfl, T. et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N. Engl. J. Med. 369, 2379–90 (2013).*
- *Nangalia, J. et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N. Engl. J. Med. 369, 2391–405 (2013).*

02 Intended use

The Imegen® CALR kit can be used to detect insertions/deletions (indels) in the CALR gene by PCR and subsequent capillary electrophoresis.

PCR products are separated by capillary electrophoresis and detected by 6-carboxyfluorescein (6-FAM) labeling.

Imegen® CALR kit has been designed for in vitro diagnostics and it is directed to professionals from the molecular biology sector.

03 Technical characteristics

This kit has been validated using biobank samples, samples previously diagnosed by other laboratories using different techniques, and synthetic plasmids generated from the reference sequence with three mutational variants, whose quality control provides the specific sequences included in the vectors.

The necessary material for this analysis is genomic DNA, mainly from peripheral blood. The necessary amount of DNA is 50 ng.

Following the protocol specified in section 7 (Assay protocol), the limit of detection (LOD) has been established at 5%.

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

- Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
- You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.
- Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.
- In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.
- Safety data-sheets (MSDS) of all hazardous components contained in this kit are available on request to Health in Code S.L.
- This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive not biological or environmental pollutants.
- This kit has been validated using specific equipment and conditions which might vary from the conditions in other laboratories. Thus, it is recommended that each laboratory performs an internal validation prior to the utilization of the kit.
- The manufacturer is not responsible for the malfunction of the assay when one or more 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 employs reagents not validated by Health in Code S.L., considering them equivalent to those provided in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient reagents to perform 33 determinations using the following reagents:

- **CALR Master Mix**: PCR master mix with the specific oligonucleotides, dNTPs, $MgCl_2$, and buffer required to perform the amplification reactions.
- **Taq**: Enzyme required to perform the amplification reactions.
- **Positive control**: Equimolar mixture of plasmid DNA including one normal allele, one allele with an insertion, and two alleles with deletions.

Reagents	Color indicator	Quantity	Conservation
CALR Master Mix	White cap	2 x 408 µL	-20°C
Taq	Yellow cap	25 µL	-20°C
Positive Control	White cap	33 µL	-20°C

Table 1. Components of the Imegen® CALR kit

06 Equipment, reagents and material not included in the kit

Equipment:

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

Reagents:

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

Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 1000µL)
- Sterile 1.5 mL tubes
- 0.2 mL tubes
- 96-well plates compatible with capillary electrophoresis equipment
- Sealing film for 96-well plates
- Latex gloves

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

Complementary kits

Health in Code S.L also offers the real-time PCR Imegen® MPL (ref IMG-236) kit for the diagnosis of myeloproliferative neoplasms.

07 Assay protocol

07.1 | Preparation of the PCR assay

The Imegen® CALR is designed to perform one PCR action for each sample to be analyzed. Moreover, analyzing a negative control for each amplification cycle is recommended to rule out contamination of PCR reagents, as well as a positive control.

In order to estimate the necessary amount of reagents, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend performing the calculations either considering one extra reaction or increasing the volume of each reagent by 10%.

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

01 Thaw the *CALR Master Mix* tube, the positive control, and the DNA samples. Vortex each reagent and keep cold.

02 In a 1.5 mL tube, prepare the reaction mix by adding 22.5 µL of *CALR Master Mix* and 0.5 µL of *Taq* for each sample or control. Mix on a vortex and spin.

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

04 To 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 for the negative PCR control, and 2 µL of the positive control.

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

↘ Optimal program:

Parameters	Phase 1	Phase 2			Phase 3	
No. of cycles	1x cycles	30x cycles			1x cycles	
		Denaturation	Annealing	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 the devices T3 (Biometra), SimpliAmp Thermal Cycler and GENEAMP® PCR System 2720 (Applied Biosystems)

↘ The protocol can be stopped at this point. The 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 fragment plate

To perform capillary electrophoresis, PCR products (fragments) must be prepared in a 96-well plate that is compatible with the capillary electrophoresis equipment, as indicated below:

01 Add the following reagents to a 1.5 mL tube:

Reagent	Amount per reaction
Formamide	18 µL
GeneScan™ 500 LIZ dye	0.5 µL

In case several reactions are to be performed, the volume of each reagent should be increased by 10%.

NOTE: The volume of sizing dye can be increased or decreased to adjust peak intensity on electropherogram.

02 Dispense 18.5 µL of the previous mix into each well.

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

NOTE: Sample volume can be increased or decreased (by diluting the samples with nuclease-free water) to adjust peak intensity on electropherogram.

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

05 Store the plate at 4°C until it is introduced in the capillary electrophoresis equipment.

07.3 | Capillary electrophoresis

Once the fragment plate has been prepared, the reactions must be subjected to capillary electrophoresis. Manufacturer-recommended electrophoresis parameters for the specific sequencer model used must be applied.

To set up these parameters, it must be noted that the amplification range will vary between 100 and 500 pb; that 6-FAM -labeled primers are used, and that molecular weight pattern is labeled with *GeneScan™ 500 LIZ dye*.

The image below shows the parameters optimized for a 3730xl DNA Analyzer sequencer (ThermoFisher Scientific) using polymer *POP-7™*.

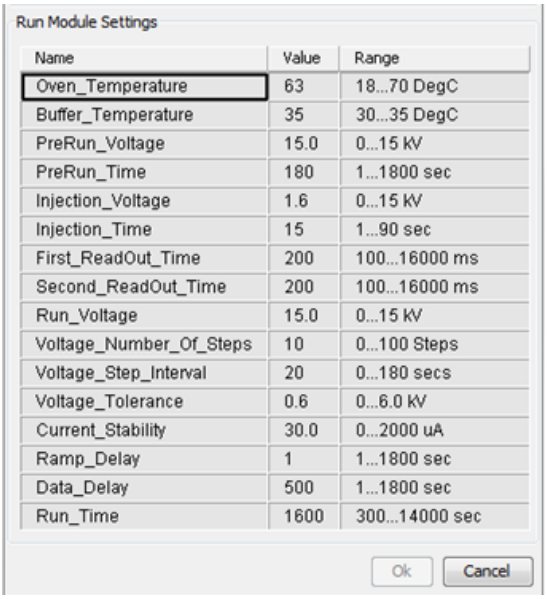

Figure 1. Parameters optimized for a 3730xl DNA sequencer

Detection intensity can vary among different devices depending on the model, the status of the device's optical system, and the voltage injection. For this reason, increasing or decreasing the amounts of sizing dye or PCR product may be required to perform capillary electrophoresis.

08 Analysis of results

The results are analyzed by specifically designed fragment analysis software based on the resulting file after capillary electrophoresis. The result is an electropherogram showing peaks with certain intensity levels (height) and at a distance that is directly proportional to fragment size (see figure 2).

The image below shows examples of possible results:

*Figure 2. PCR results. **A** Profile of a DNA sample from a disease-free donor (232 pb). **B** and **C** Profiles of three DNA samples from patients carrying the two most frequent CALR mutations (5 bp insertion and 52 bp deletion, respectively).*

Below is an example of the results obtained for the positive control and its size (in bp). This control includes four alleles: one normal (non-mutated) allele, one allele with a 5-bp insertion (c.1154-1155insTTGTC), one allele with a 52-bp deletion (c.1092-1143del), and one allele with a 1-bp deletion (c.1122del) according to NM_004343.3.

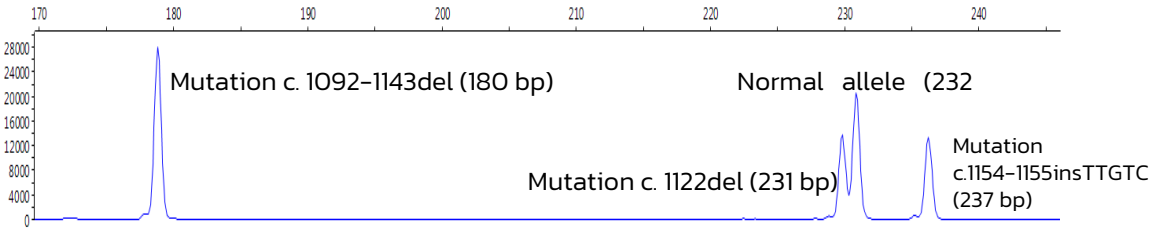

Figure 3. Electropherogram obtained for the positive control.

09 Troubleshooting

The following table shows possible results for the analyzed samples, the positive control, the sizing dye, and the negative control. If an unexpected result is obtained, the interpretation of the result and the most likely reason for the result are summarized in the following table:

Problem	Analyzed samples	Control Positive	Sizing dye	Negative Control	Result / Interpretation
Weak or absent fluorescence signal				√	Expected result
	√			√	Insufficient DNA amount and/or quality[1] Impure template DNA[2]
	√	√	√	√	Capillary electrophoresis failure[3] Denaturation failure[4]
	√	√		√	PCR failure[5]
Excessive fluorescence signal	√				Excessive DNA amount [6]
	√	√			
Presence of more peaks than expected		√		√	Contamination[7]

Table 3. Interpretation the possible results obtained using the Imegen® CALR kit.

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

(2) Impure template DNA: High salt concentrations or altered pH values can inhibit PCR. If the template DNA used is dissolved in elution tampon with pH different from 8 or with high EDTA concentrations, the volume of DNA should not exceed 20% of the total reaction volume. Remaining residues of the reagents used during extraction can also affect the PCR reaction. If this is the case, clean the DNA or prepare a new extraction.

(3) Capillary electrophoresis failure: Verify whether the equipment settings match those specified, then reinject the samples.

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

(5) PCR failure: Verify that you are using the indicated PCR program.

(6) Excessive DNA amount: Make sure that the amount of DNA used is adequate. If this is the case, dilute the PDC product in sterile deionized water, perform denaturation again, and load into the sequence again.

(7) Contamination: It can be due to another template DNA or to previously amplified DNA. Cross-contamination can lead to false positives or false negatives, resulting in issues with result interpretation. Use filtered pipette tips and change gloves regularly.

10 Limitations

10.1 | Equipment

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

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

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.

Imegen® CALR 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. If a different device is used, the specifications in the protocol of the corresponding platform should be followed.

10.2 | Reagents

Imegen® CALR 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).

For capillary electrophoresis, it is advised to use the reagents recommended by the sequencer manufacturer: **ThermoFisher Scientific**.

Should you have any questions, please contact Health in Code's technical support team

10.3 | Product stability

The optimal performance of this product is achieved when the specified recommended storage conditions are applied, within the product expiration date associated with each batch.

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

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