Instructions for use

Imegen® Quimera qPCR

Ref. IMG-116

CE IVD

Manufactured by:

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

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Health in Code guarantees that its products are free of defects, both in 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 provides no other 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 products or reimburse the price thereof, at the client's choice, provided that, however, materials or workmanship prove to be defective.

Health in Code shall not be liable for any loss or damage, whether direct or indirect, resulting in economic loss or harm incurred as a result of use of the product by the buyer or user.

All Health in Code products undergo strict quality control. The Imegen®-Quimera kits have 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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Instructions for Use (IFU) modifications		
Version 03	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 02	SEP 2022	Change of the manufacturer's identification, going from Imegen to Health in Code S.L.
Version 01	MAY 2022	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

The analysis of molecular chimerism resulting from allogenic hematopoietic cell transplantation has become an established method to follow transplant progression, since it offers accurate and valuable information that allows targeting post-transplant treatments or interventions with the purpose of anticipating any potential risk of relapse, rejection, or graft-versus-host disease. This approach is highly useful not only to determine the risk of relapse, rejection, or graft-versus-host disease, but also to assess the response to different treatment modalities.

The whole Imegen®-Quimera kit family has been developed in collaboration with the Carlos Haya Regional Hospital (Málaga, Spain), included in the Andalusian regional Public Healthcare Service (Servicio Andaluz de Salud) (SAS). As a result of this agreement, Health in Code holds the **exclusive worldwide license** on the know-how of the products for their manufacturing and commercial exploitation.

References

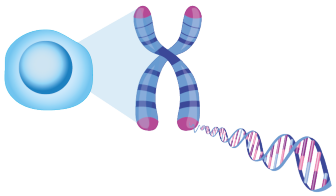
› *Jiménez-Velasco A, Barrios M, Román-Gómez J, Navarro G, Buño I, Castillejo J, et al. Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. Leukemia. 2005; 1-8.*

Procedure for hematopoietic chimerism analysis:

1. EXTRACTION OF GENOMIC DNA

1h

Genomic DNA extracted from peripheral blood or bone marrow samples.



2. SCREENING FOR INFORMATIVE POLYMORPHISMS

2h30'

A genotyping assay allows identifying an informative polymorphism suitable for patient follow-up.

3. MARKER SELECTION FOR PATIENT FOLLOW-UP

10'

In hematopoietic stem cell transplant cases, a polymorphism is considered informative when detected in the recipient and not in the donor

MARKER		RECIPIENT	DONOR	INFORMATIVE
Q116-6I	[FAM]	+ −	+ −	✗
Q116-3I	[VIC]	+ −	+ −	✗
Q116-7I	[FAM]	+ −	+ −	✓
Q116-12D	[VIC]	+ −	+ −	✗

4. QUANTIFICATION OF FOLLOW-UP MARKER

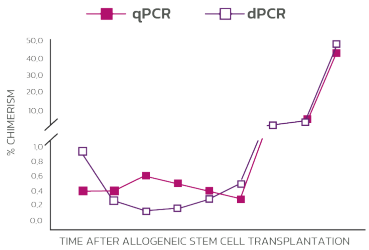
dPCR 4h
qPCR 2h30'

Molecular chimerism is quantified based on the number of copies of the informative marker relative to the number of copies of the reference gene (β-globin).

5. PATIENT FOLLOW-UP FOR HEMATOPOIETIC CHIMERISM

10'

During follow-up, hematopoietic chimerism values are plotted in a graph to study the transplant patient's progression over time.



02 Intended use

The Imegen®-Quimera kit allows determining the presence of hematopoietic chimerism during the post-transplant period through relative quantification of a molecular marker by real-time PCR. This assay is based on a previous screening for informative polymorphisms, including INDELs (insertions/deletions) and null alleles, which are used to quantify molecular chimerism as the relative amount of the informative marker with respect to total genomic DNA in the sample. To carry out this quantification, it is necessary to test for a reference gene (β-globin), which at the same time works as a positive control for the amplification and an informative polymorphism.

Therefore, it is essential to first select the informative polymorphisms for each transplant case. For this purpose, Health in Code has validated two references: Imegen®-Quimera Screening Multiplex I (IMG-116-24) and Imegen®-Quimera Screening Multiplex II (IMG-116-74). In the context of bone marrow transplants, a polymorphism is considered informative when detected in the transplant recipient but not in the donor.

Once an informative marker has been selected, it can be used for the follow-up of the transplanted patient. For this purpose, 34 markers have been developed, including INDELs and null alleles, which are listed in the table below:

Markers	Insertion (Allele +)	Deletion (Allele -)	Kit reference
SRY	X		IMG-116-2
Q116-3I	X		IMG-116-3
Q116-4I	X		IMG-116-4
Q116-5I	X		IMG-116-5
Q116-6I	X		IMG-116-6
Q116-7I	X		IMG-116-7
Q116-8I	X		IMG-116-8
Q116-9I	X		IMG-116-9
Q116-10I	X		IMG-116-10
Q116-11I	X		IMG-116-11
Q116-32I	X		IMG-116-12
Q116-31I	X		IMG-116-13
Q116-30D		X	IMG-116-14
Q116-29D		X	IMG-116-17
Q116-27D		X	IMG-116-18
Q116-12I	X		IMG-116-20
Q116-4D		X	IMG-116-21

Q116-5D		X	IMG-116-23
Q116-10D		X	IMG-116-16
RhD	X		IMG-116-75
Q116-20I	X		IMG-116-76
Q116-12D		X	IMG-116-77
Q116-23I	X		IMG-116-78
Q116-33I	X		IMG-116-79
Q116-37I	X		IMG-116-80
Q116-38I	X		IMG-116-70
Q116-44I	X		IMG-116-66
Q116-43I	X		IMG-116-81
Q116-49I	X		IMG-116-82
Q116-39I	X		IMG-116-83
Q116-50I	X		IMG-116-84
Q116-46I	X		IMG-116-73
Q116-47I	X		IMG-116-88
Q116-32I	X		IMG-116-87

Table 1. References of the Imegen®-Quimera kit for real-time PCR

These instructions for use are suitable for testing any of the 34 markers included in Table 1, as their operation is optimal under the same PCR settings. Therefore, this technique allows for rapid and effective simultaneous testing for multiple polymorphisms.

Imegen®-Quimera kits are intended solely for research use and are aimed at professionals working in molecular biology.

03 Technical characteristics

Imegen®-Quimera consists of a real-time PCR test that allows quantifying the number of copies of an informative marker for follow-up of hematopoietic chimerism. This kit uses a combination of specific primers and fluorescent hydrolysis probes to quantify either the relative amount of an informative marker with respect to the reference gene, β-globin.

Quantitative analysis of chimerism involves a relative calculation system that implements the method developed by Pfafl, which takes into account the efficiency of the amplification system and the cycle threshold (Ct) values obtained for an endogenous control, or calibrator, included in the kit as a positive control. From these values, the system allows calculating chimerism for each run. Moreover, Health in Code has developed a calculation tool to enable chimerism analysis by real-time PCR.

The type of sample used to validate these Imegen®-Quimera kits was genomic DNA from peripheral blood or bone marrow samples from patients who have undergone allogeneic stem cell transplant. The limit of quantification (LoQ) has been determined to be 0.1%, while the limit of detection (LoD) has been established at 0.01% when genomic DNA samples are used.

This product is compliant with the quality specifications of the **ISO 9001** standards regarding manufacturing materials.

04 Safety warnings and precautions

- Strictly follow the instructions of 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 abundant water.
- Safety data-sheets (MSDS) of all hazardous components contained in this kit are available on request.
- This product requires the handling of samples and materials of human origin. You should consider all materials of human origin as potentially infectious and handle 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 verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for the malfunction of the assay when the reagents included in the kit are replaced with other reagents not supplied by Health in Code.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code but are considered by the user equivalent to those provided in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient freeze-dried reagents to carry out 25 digital PCR determinations:

- **Polymorphism Master Mix**: Oligonucleotides and probe necessary to amplify the target marker (polymorphism). The probe used to detect the marker is labeled using FAM™.
- **β-globin Master Mix**: Oligonucleotides and probe necessary to amplify the reference gene (β-globin). The probe used to detect the marker is labeled using FAM™.
- **Positive control**: A standard that acts as a calibrator and allows for relative quantification of markers and for monitoring of transplant progression without needing to collect and store a large amount of sample from the recipient prior to transplantation. This standard is a synthetic plasmid that contains each polymorphism in homozygosis. Therefore, although this is not its main purpose, it also provides information about whether the polymorphism is found in the sample in homozygosity (result=expected percentage) or in heterozygosity (result = half the expected percentage).

Reagents	Quantity	Conservation
Polymorphism Master Mix	25 rxn	4°C
β*-globin *Master Mix	25 rxn	4°C
Positive control	25 rxn	4°C

Table 2. Components of the Imegen®-Quimera kit for real-time PCR

06 Equipment, reagents and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler
- Micropipettes (10 µL, 20 µL, and 200 µL)
- Vortex mixer

Reagents:

- Nuclease-free water
- Master Mix 2X (HotStart DNA polymerase)

Materials:

- Optical 96-well plates or 0.2 ml optical tubes
- Optical film for 96-well plates or optical lids for 0.2 ml tubes
- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 ml tubes
- Powder-free latex gloves

Complementary kits

Before quantifying an informative marker, the previous step consists of determining the informativity of the possible polymorphisms by real-time PCR; for this purpose, Health in Code has developed the kits Imegen® Quimera Screening Multiplex I (Ref. IMG-116-24) and Imegen® Quimera Screening Multiplex II (Ref. IMG-116-74)

07 Assay protocol

07.1 | Reagent preparation

The reagent included in this kit is freeze-dried. Before applying the protocol, the first step is to rehydrate the reagents by adding nuclease-free water. To enable resuspension, it is recommended to shake and spin the tubes containing the reagents and store them at 4 °C for one hour before their use.

Reagents	Rehydration
Polymorphism Master Mix	80 µL of water/vial*
β-globin *Master Mix*	80 µL of water/vial*
Positive control	100 µL of water/vial*

Table 3. Rehydration volume for the components of the kit

(*) If these reagents are not to be used immediately after rehydration, storage at -20 °C is recommended.

07.2 | Preparation of amplification reactions

The assay must include the following reactions:

- Reactions of each sample in duplicate.
- Positive control reactions in duplicate.
- Negative control reaction (a reaction that contains water instead of DNA to confirm the absence of contamination during the process).

Relative quantification using the kits in the Imegen®-Quimera series requires the preparation of two different PCR mixes: one to test for the reference gene (β-globin) and one to test for the informative polymorphism.

The protocol for preparing the amplification reactions is detailed below:

01 Thaw the necessary reagents to perform the analysis:

- Genomic DNA samples diluted to the optimal concentration (25 ng/µL).
- Polymorphism Master Mix (rehydrated).
- β-globin Master Mix (rehydrated).
- Nuclease-free water for negative controls.
- Real-time PCR 2X Master Mix (not included).

02 Vortex and spin the reagents.

03 Prepare two PCR mixes by adding the indicated amount of each reagent to 1.5 mL tubes according to the total number of reactions. 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.

Reagent	Amount per reaction
Polymorphysm Master Mix	3 µL
PCR 2X Master Mix	5 µL

Reagent	Amount per reaction
β-Globin Master Mix	3 µL
PCR 2X Master Mix	5 µL

04 Vortex the PCR mixes and dispense 8 µL to the corresponding PCR wells or tubes.

05 Dispense 2 µL of the DNA sample (100 ng/µL), of positive control, or of nuclease-free water (negative control) to the corresponding wells.

↘ Low sample concentration

The first sample collected after transplantation sometimes results in low genomic DNA concentration. If so, we recommend preparing amplification reagents using the following amounts of the reagents.

Reagent	Amount per reaction
β-globin Master Mix or polymorphism Master Mix	5 µL
2x PCR Master Mix	7 µL
DNA	10 µL

Table 4. Amounts of the different reagents required for samples with low concentrations

07.3 | Settings for the real-time PCR program

Depending on the equipment used to perform real-time PCR, the instructions below must be followed to set up the amplification program.

7500 Fast o StepOne Real-Time PCR system (Thermo Scientific)

- **Type of experiment**: Quantitation —Standard curve
- **Ramp rate**: Standard
- **Reaction volume**: 10 µL
- **ROX™ baseline reference**: included
- **Fluorophores of TaqMan® probes**:

Probe	Recipient	Quencher
β-globin	FAM™	MGB
Informative polymorphism	FAM™	MGB

Table 5. Information about probes

◇ Optimal program:

Fields	Phase 1 Enzymatic activation	Phase 2 PCR	
No. of cycles	1 initial cycle	50 cycles	
		Denaturation	Oligonucleotide binding/extension
Temperature	95°C	95°C	58°C
Time	10 minutes	15 seconds	1 minute*

Table 6. Optimal PCR program for the 7500 FAST or StepOne Real-time PCR systems

(*) Fluorescence detection

Lightcycler 480 (Roche)

◇ Optimal program:

Fields	Phase 1 Enzymatic activation	Phase 2 PCR			Phase 3
No. of cycles	1 initial cycle	50 cycles			1 final cycle
		Denaturation.	Primer binding	Extension	
Temperature	95°C	95°C	58°C	72°C	40°C
Time	10 minutes	5 seconds	10 seconds	15 seconds*	20 seconds

Table 7. Optimal PCR program for Lightcycler 480

(*) Fluorescence detection

08 Analysis of results

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

- Verify that no amplification is found in negative controls. If amplification is detected, it is recommended to repeat the test to rule out accidental contamination.
- Verify that the reference gene (β-globin) is detected both for the standard and the samples at a PCR cycle lower than 26. Amplifications over this Ct suggest that the amount of DNA in the sample is not sufficient, which will result in decreased sensitivity of the test performed.
- A specific software (**Quimera Software**) has been developed for relative quantification of haematopoietic chimera.
- This software will deliver the result once the following parameters have been entered:
  - Select the polymorphism used for the analysis.
  - Indicate the Ct for the standard or calibrator, both for β-globin and for the target informative polymorphism.
  - Indicate the Ct for the samples, both for β-globin and for the target informative polymorphism.
- Use the following equation to perform the calculations manually:

$$\%\ \boldsymbol{Chimera} = \frac{\left(\boldsymbol{E_{target}}\right)^{\boldsymbol{Ct\ target\ (calibr.-sample)}}}{\left(\boldsymbol{E_{ref}}\right)^{\boldsymbol{Ct\ ref\ (calibr.-sample)}}} \boldsymbol{x\ 100}$$

E = Reaction efficiency
***Target** = Target informative polymorphism*
***Reference** = β-globin*

↘ Imegen®-Quimera Software, by Health in Code

Health in Code has designed and developed a user-friendly application that allows creating a patient database, as well as recording screening results for informative polymorphisms, their quantification in the different follow-up samples from a patient, and the medical actions taken in regard to said patient during follow-up. Moreover, the user can plot all the medical actions and the patient's progression and can export the results.

A video tutorial on how to use our **Imegen®-Quimera** application is available at the following link: youtu.be/K38cV3hacm8

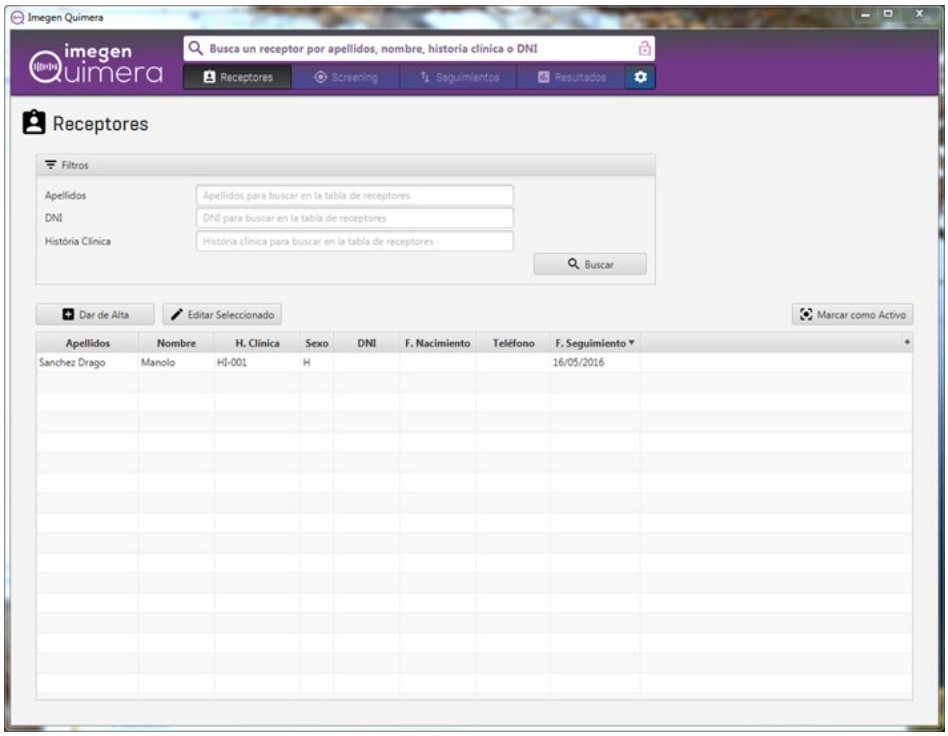


Image 1. View of the patient follow-up application developed by Health in Code

09 Troubleshooting

The table below summarizes the possible test results that can be obtained for the different controls and one sample in one run, along with their interpretation:

Control	Polymorphism	β-Globin	Cause
DNA sample	+	+	Expected result
	–	+	Expected result when testing for an informative marker
	–	–	Failure of sample amplification[1]
Positive control	+	+	Expected result
	–	–	Incorrect PCR settings[2]
Negative PCR control	–	–	Expected result
	+	+	Human DNA contamination[3]

Table 8. Interpretation of the possible results obtained using Imegen-Quimera

(1) Sample amplification failure: Verify that DNA concentration and quality meet the requirements; if so, the result may be caused by a highly degraded sample. In this case, it a new analysis or DNA extraction should be performed before interpreting the results.

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

(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.

Other issues that may arise include:

- **The resulting amplification curves are not ROX-normalized:**

  Verify that the ROX fluorochrome has been set as a passive reference to normalize the variations caused by pipetting or equipment in each well.

- **Low reproducibility among samples:**
  - **Pipetting errors**

    Proposed solution: Avoid variations in pipetting by using gloves, filtered tips, and properly calibrated pipettes.

  - **Variations caused by PCR masters**

    Proposed solution: Prepare a sufficient amount of PCR master to prepare all the required reactions and make sure that the master is well homogenized before use (the use of a vortex is recommended).

- **Concentración del ADN diana cercana al límite de detección**: La reproducibilidad entre réplicas se puede ver comprometida si el ADN diana se encuentra a una concentración cercana al límite de detección.
- **Target DNA concentration close to the limit of detection**: reproducibility among replicates may be compromised when target DNA is found at concentrations near the limit of detection.

+ **Low-intensity or undetected signal**:
  - **One or more of the components has not been added to the reaction or its concentration is not adequate.**

    Proposed solution: Prepare the master mixes again and repeat the assay if necessary.

  - **Fluorescent reading failure**

    Proposed solution: Verify that the fluorescence signal has been captured in the appropriate step and channel. Confirm that the probes have been configured in the amplification program associated with the correct fluorochrome and that the RXO/fluorescence levels are correct.

  - **Presence of inhibitors**

    Proposed solution: Inhibitors from the extraction process can sometimes hinder or prevent the amplification reaction. If so, DNA can be purified or diluted to remove or minimize the presence of inhibitors in the reaction.

  - **Suboptimal PCR conditions**

    Proposed solution: Verify that the amplification conditions are adequate.

  - **Problems with baseline and/or threshold**

    Proposed solution: Manually set the baseline and/or threshold values to obtain accurate Cp/Ct values.

10 Limitations

10.1 | Equipment

Imegen®-Quimera has been validated for use with the following real-time PCR platforms:

- 7500 FAST Real-Time PCR System (ThermoFisher Scientific)
- StepOne Plus Real-Time PCR System (ThermoFisher Scientific)
- LightCycler 480 Real-Time PCR System (Roche)

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 team.

10.2 | Reagents

We recommend using the PCR reagents indicated by the manufacturer of the thermal cycler to be used for real-time PCR assays. Should you have any questions, please contact our technical support team.

Using a HotStart Polymerase (No Fast) is recommended.

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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