Instructions for use

Imegen® Quimera Screening Multiplex Plus

Ref. IMG-116-26

CE IVD

Manufactured by:
HEALTH IN CODE, S.L.
Calle de la Travesía s/n, 15E Base 5, Valencia, 46024, Spain
+34 963 212 340 - info@healthincode.com

healthincode.com

Code: HIC-PT-KIT 03-F-03 V.04

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This product is designed for *in vitro* diagnostics. Health in Code, S.L. makes no other express or implied guarantee that 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 is to replace the products or refund the purchase price, 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. The Imegen® Quimera Screening Multiplex Plus kit has passed all internal validation tests, which guarantee the reliability and reproducibility of each batch manufactured.

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

+34 963 212 340

tech.support@healthincode.com

Modifications to the instructions for use (IFU)		
Version 13	MAY 2024	New clarifications in Section 9: "Troubleshooting".
Version 12	DEC 2023	Review and update of document content
Version 11	JUL 2023	The enzyme is renamed in sections 06, 07.1 and 10.2. Clarification of the use of RhD and SRY markers in sections 02 and 03. Modification of table 8 in section 09 "Troubleshooting". Section 11 "Performance characteristics" is added.
Version 10	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 09	SEP 2022	Change of manufacturer's identification: from Imegen to HEALTH IN CODE, S.L.
Version 08	NOV 2019	Update of section 9. Troubleshooting
Version 07	NOV 2019	Update of section 9. Troubleshooting
Version 06	OCT 2018	Specification in section 3 of the amount of DNA required to carry out the analysis.

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01 General information

The analysis of molecular chimerisms resulting from allogeneic transplantation is now a well-established method for transplant follow-up, as it provides precise, valuable information to orient post-transplant treatment or intervention, with the aim of anticipating possible risks of relapse, rejection or graft-versus-host disease. The approach is extremely useful not only to determine the risk of relapse, rejection or graft-versus-host disease, but also to assess the response to different forms of treatment.

The entire Imegen® Quimera kit family has been developed in collaboration with the Málaga Regional University Hospital within the Andalusian Health Service (SAS). As a result of this agreement, Health in Code, S.L. has an **exclusive, worldwide license** for the know-how of the products for their manufacture and exploitation.

References

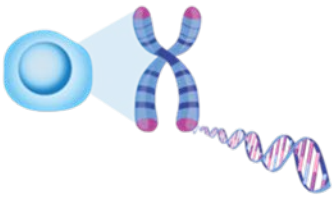
- *Jiménez-Velasco A, Barrios M, Román-Gómez J, Navarro G, Buño I, Castillejo J, et al. Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. Leukemia. 2005 Mar;19(3):336-43. doi: 10.1038/sj.leu.2403622. PMID: 15674363.*
- *Stahl T, Böhmeb MU, Krögera N, Fehse B. Digital PCR to assess hematopoietic chimerism after allogeneic stem cell transplantation. Experimental Hematology. 2015 Jun;43(6):462-8.e1. doi: 10.1016-j.exphem.2015.02.006. Epub 2015 Mar 18. PMID: 25795523*

Hematopoietic chimerism analysis procedure:

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

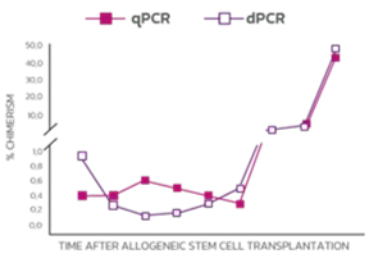
dPCR 4h
qPCR 2h30'

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 Screening Multiplex Plus kit allows the selection of informative markers for the follow-up of bone marrow transplant patients by simultaneous analysis of 16 insertion/deletion polymorphisms (INDELs) in 8 independent reactions by multiplex real-time PCR.

In cases of hematopoietic cell transplantation, a polymorphism is considered informative when detected in the transplant recipient and not in the donor, whereas, in solid organ studies, a marker would be considered informative if detected in the donor and not in the recipient.

To determine the informativity of polymorphisms, the Imegen® Quimera Screening Multiplex Plus (IMG-116-26) and Imegen® Quimera Screening Multiplex Plus II (IMG-116-25) kits have been developed. Table 1 lists the markers included in both kits.

The Imegen® Quimera Screening Multiplex Plus kit is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

The Imegen® Quimera Screening Multiplex Plus kit consists of a genotyping assay of 16 biomarkers, including null alleles and INDELs (insertion deletion) that allows the identification of informative markers for the analysis of hematopoietic chimerisms. The presence or absence of these polymorphisms is determined using a combination of oligonucleotides and fluorescent hydrolysis probes specific for each of the 16 markers in multiplex PCR systems (Table 1).

The Imegen® Quimera Screening Multiplex Plus kit does not contain the SRY and RhD markers but they are available for follow-up with the Imegen® Quimera dPCR Dry kit, since, the SRY marker, present in the Chr Y, is informative when the recipient is male and the donor is female and the RhD when the recipient has Rh+ blood group and the donor Rh-. In the absence of this baseline clinical data, the RhD marker has been included in the Imegen® Quimera Screening Multiplex Plus II kit.

The Imegen® Quimera Screening Multiplex Plus and Imegen® Quimera Screening Multiplex Plus II kits, together with the SRY marker, present on the Chr Y, include a total of 33 markers (Figure 1 and Table 1).

↘ 33 markers on 18 chromosomes

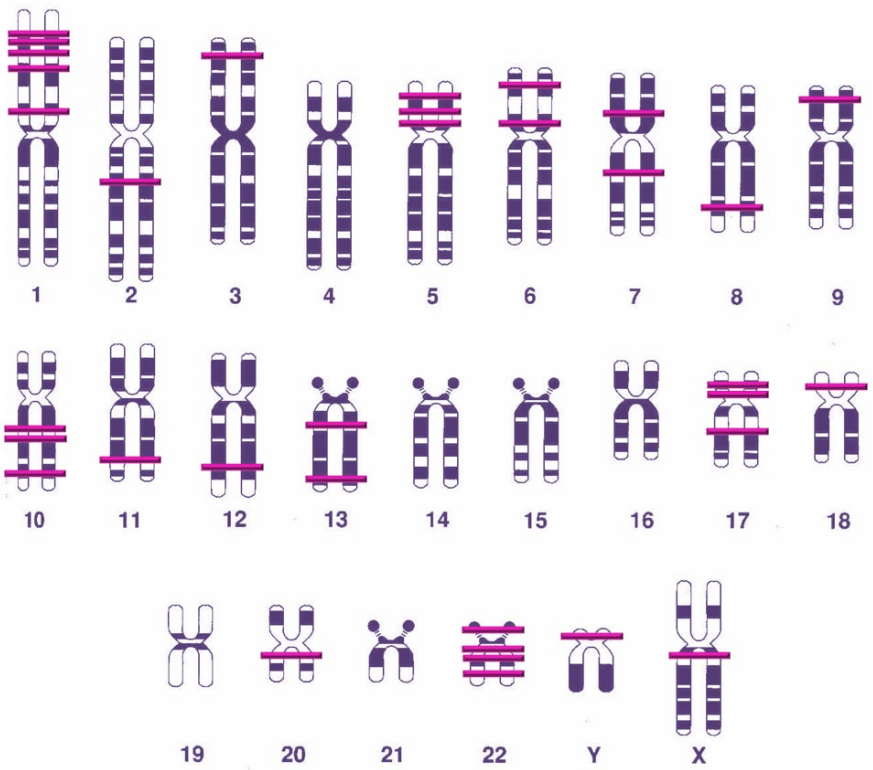


Figure 1. Chromosomal representation of biomarkers included in genotyping and chimerisms monitoring assays.

The material needed for this study is genomic DNA mainly from peripheral blood. The total amount of DNA needed is 450 ng of pre-transplant recipient sample and 450 ng of donor sample.

IMG-116-26 Screening Multiplex Plus

Biomarker Name	Chromosomal Position
31	20q11.22
61	10q26.2
12D	5p13.2
71	Xq28
111	1p13.3
51	10q21.2
41	17p13.2
101	22q13.32
231	13q34
281	12q24.23
321	3p25.3
311	6p21.2
29D	17q21.31
30D	7q21.3
27D	18p11.22
241	1p34.1

IMG-116-25 Screening Multiplex Plus II

Biomarker Name	Chromosomal Position
331	1p36.13
371	5p15.32
381	6p12.3
441	13q14.11
431	12q24.21
491	2q21.2
391	7p12.3
501	1p36.11
451	15q21.3
91	22q11.22
411	10q23.33
201	8q24.22
461	9p23
471	11q23.2
421	11q22.3
RhD	1p36.11

Table 1. Chromosomal position of the biomarkers. The SRY marker, located on Chr Y, is also available for follow-up.

The clinical performance of this kit has been validated using genomic DNA extracted from peripheral blood or bone marrow of human samples. The detection limit has been set at 0.01%.

The cumulative informativity of this panel, together with the SRY and RhD markers, is 99.1%. In case of analyzing also the panel of markers included in Imegen® Quimera Screening Multiplex Plus II, the cumulative informativity is 99.98%.

04 Safety warnings and precautions

- We recommend strictly following the instructions in this manual, especially regarding the handling and storage conditions for 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. We recommend 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 or 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 liable 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 to analyze 10 different genomic DNA samples, or 5 recipient/donor cases.

The kit consists of a strip of 8 tubes containing one Screening Master in each tube. Each Master consists of two pairs of oligonucleotides and two TaqMan®-MGB probes with different labeling (FAM™ or VIC™) for the simultaneous analysis of two different polymorphisms.

Tube	Reactions	Markers	Storage	Rehydration*
1	10 reactions	Q116-6I Q116-3I	4°C	33 µL of water/vial
2	10 reactions	Q116-7I Q116-12D	4°C	33 µL of water/vial
3	10 reactions	Q116-11I Q116-5I	4°C	33 µL of water/vial
4	10 reactions	Q116-4I Q116-10I	4°C	33 µL of water/vial
5	10 reactions	Q116-23I Q116-28I	4°C	33 µL of water/vial
6	10 reactions	Q116-32I Q116-31I	4°C	33 µL of water/vial
7	10 reactions	Q116-30D Q116-29D	4°C	33 µL of water/vial
8	10 reactions	Q116-27D Q116-24I	4°C	33 µL of water/vial

Table 2. Imegen® Quimera Screening Multiplex Plus kit components and storage temperature

(*) Once rehydrated, the reagents should be stored at -20°C.

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
- Spectrophotometer (*NanoDrop*; Thermo Fisher Scientific)

Reagents:

- Nuclease-free water
- PCR Hot Start Master Mix (TaqMan™ Environmental Master Mix 2.0, Thermo Fisher Scientific)

Materials:

- 96-well optical plates or 0.2 mL optical tubes
- Optical film for 96-well plates or optical caps for 0.2 mL tubes
- Pipette tips with filter (10 µL, 20 µL and 200 µL)
- 1.5 mL sterile tubes
- Powder-free latex gloves

Complementary kits

In case the Imegen® Quimera Screening Multiplex Plus kit does not identify any informative markers, we recommend using the Imegen® Quimera Screening Multiplex Plus II kit **(Ref. IMG-116-25)**, which offers 16 alternative markers.

Once a polymorphism has been identified as informative, we recommend purchasing the corresponding Imegen® Quimera dPCR kit from our catalog for patient follow-up and thus analysis of the transplanted organ and assessment of the risk of relapse. The Imegen® Quimera kits allow absolute quantification of the amount of informative marker (chimerism) or relative quantification in relation to the total amount of genomic DNA, using a reference gene (β-globin). The reference gene is analyzed simultaneously with the informative marker in a multiplex reaction, and also serves as a control of the quality and quantity of the analyzed DNA sample.

Kit name	Reference
Imegen® Quimera SRY dPCR	IMG-116-27
Imegen® Quimera RhD dPCR	IMG-116-28
Imegen® Quimera Q116-3I dPCR	IMG-116-29
Imegen® Quimera Q116-4I dPCR	IMG-116-30
Imegen® Quimera Q116-5I dPCR	IMG-116-31
Imegen® Quimera Q116-6I dPCR	IMG-116-32
Imegen® Quimera Q116-7I dPCR	IMG-116-33
Imegen® Quimera Q116-11I dPCR	IMG-116-34
Imegen® Quimera Q116-10I dPCR	IMG-116-35
Imegen® Quimera Q116-12D dPCR	IMG-116-36
Imegen® Quimera Q116-23I dPCR	IMG-116-37
Imegen® Quimera Q116-24I dPCR	IMG-116-38
Imegen® Quimera Q116-20I dPCR	IMG-116-40
Imegen® Quimera Q116-27D dPCR	IMG-116-41
Imegen® Quimera Q116-28I dPCR	IMG-116-42
Imegen® Quimera Q116-29D dPCR	IMG-116-43
Imegen® Quimera Q116-30D dPCR	IMG-116-44
Imegen® Quimera Q116-31I dPCR	IMG-116-45
Imegen® Quimera Q116-32I dPCR	IMG-116-46
Imegen® Quimera Q116-33I dPCR	IMG-116-47
Imegen® Quimera Q116-9I dPCR	IMG-116-48
Imegen® Quimera Q116-37I dPCR	IMG-116-49
Imegen® Quimera Q116-38I dPCR	IMG-116-50
Imegen® Quimera Q116-39I dPCR	IMG-116-51
Imegen® Quimera Q116-41I dPCR	IMG-116-52
Imegen® Quimera Q116-42I dPCR	IMG-116-53
Imegen® Quimera Q116-43I dPCR	IMG-116-54
Imegen® Quimera Q116-44I dPCR	IMG-116-55
Imegen® Quimera Q116-45I dPCR	IMG-116-56
Imegen® Quimera Q116-47II dPCR	IMG-116-57
Imegen® Quimera Q116-49I dPCR	IMG-116-58
Imegen® Quimera Q116-50I dPCR	IMG-116-59
Imegen® Quimera Q116-46II dPCR	IMG-116-60

Table 3. Imegen® Quimera dPCR kits for digital PCR follow-up

07 Assay protocol

07.1 | Preparation of reagents

All reagents included in the kit are lyophilized. The first step before using any of our kits will be to rehydrate the reagents by adding 33 µL of nuclease-free water/vial*. In order to facilitate the resuspension of each component, it is recommended to shake and spin the tubes containing the reagents and store them at 4°C for one hour before use.

(*) If these reagents are not going to be used after rehydration, it is recommended to keep them at -20°C.

07.2 | Preparation of amplification reactions

The assay should include the following reactions:

- Reactions with the recipient sample.
- Reactions with the donor sample.

Simultaneous analysis of the 16 markers with the Imegen® Quimera Screening Multiplex Plus kit requires the preparation of eight different PCR mixes. Each PCR mix will consist of:

- **Screening Multiplex Master Mix**
- **PCR Hot Start Master Mix (TaqMan™ Environmental Master Mix 2.0, Thermo Fisher Scientific) (not supplied)**

The recommended protocol for preparing amplification reactions is as follows:

01 Thaw the strip of 8 tubes containing the Screening Masters and the recipient and donor DNA.

02 Vortex each reagent and keep cold.

03 Add 45 µL of PCR Hot Start Master Mix and 18 µL of recipient DNA at 25 ng/µL (quantified by *NanoDrop*) into a 1.5 mL tube.

04 Add 45 µL of Hot Start PCR Master Mix and 18 µL of donor DNA at 25 ng/µL (quantified by *NanoDrop*) into a 1.5 mL tube.

05 Vortex and pipette 7 µL of the Master Mix with the recipient DNA into 8 wells and 7 µL of the Master Mix with the donor DNA into another 8 wells.

06 Add 3 µL of each Screening Master Mix to both wells with recipient DNA and wells with donor DNA.

07.3 | PCR program setup, loading and reading

Depending on the equipment to be used to perform the real-time PCR, the following instructions should be followed to set up the amplification program:

Tube	Markers	Insertion (Allele +)	Deletion (Allele –)	Labeling	Emitter or Quencher
1	Q116-6I	X		FAM™	MGB
	Q116-3I	X		VIC™	
2	Q116-7I	X		FAM™	
	Q116-12D		X	VIC™	
3	Q116-11I	X		FAM™	
	Q116-5I	X		VIC™	
4	Q116-4I	X		FAM™	
	Q116-10I	X		VIC™	
5	Q116-23I	X		FAM™	
	Q116-28I	X		VIC™	
6	Q116-32I	X		FAM™	
	Q116-31I	X		VIC™	
7	Q116-30D		X	FAM™	
	Q116-29D		X	VIC™	
8	Q116-27D		X	FAM™	
	Q116-24I	X		VIC™	

Table 4. Information on the probes included in the Imegen® Quimera Screening Multiplex Plus kit

7500 Fast or StepOne Plus Real-Time PCR system (Thermo Fisher Scientific)

- **Type of experiment**: Quantitation – Standard curve
- **Ramp speed**: standard
- **Reaction volume**: 10 µL
- **ROX™ baseline reference**: included
- **Fluorophores of TaqMan® probes**:
- **Optimal program**:

Fields	Stage 1 Enzymatic activation	Stage 2 PCR	
No. of cycles	1 initial cycle	50 cycles	
		Denaturation	Primer binding/extension
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	1 minute*

Table 5. Optimal PCR program for the 7500 FAST or StepOne Plus

(*) Fluorescence detection

Lightcycler 480 (Roche)

- Optimal program:

Fields	Stage 1 Enzymatic activation	Stage 2 PCR			Stage 3
No. of cycles	1 initial cycle	50 cycles			1 final cycle
		Denat.	Primer binding	Extension	
Temperature	95°C	95°C	60°C	72°C	40°C
Time	10 minutes	5 seconds	10 seconds	15 seconds*	20 seconds

Table 6. Optimal PCR program for LightCycler 480 thermal cycler

(*) Fluorescence detection

- **Analysis**: Fit points for all samples

08 Analysis of results

The analysis of the results is based on the detection of an informative polymorphism, i.e. detected in the recipient and not detected in the donor.

A table of possible results is shown below:

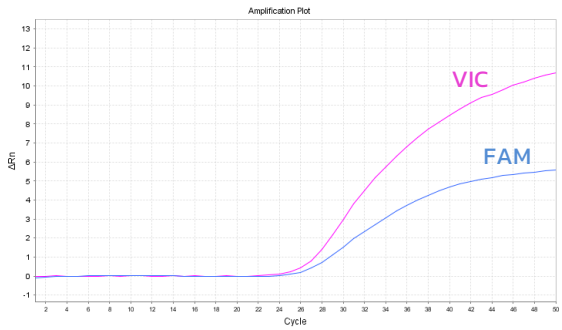
Reagents	Results		Informativity	
	Recipient	Donor	Bone Marrow	Solid organs
Polymorphism	+	+	Not informative	Not informative
Polymorphism	+	–	Informative	Not informative
Polymorphism	–	–	Not informative	Not informative
Polymorphism	–	+	Not informative	Informative

Table 7. Interpretation of possible results obtained with Imegen® Quimera Screening Multiplex Plus

In case no informative marker has been obtained, it is recommended to contact the Technical Department: tech.support@healthincode.com

Below is a figure showing the results of two multiplexed markers. The marker marked with VIC would be informative in case of bone marrow transplantation, but not in solid organ transplantation, as shown in Table 7.

Recipient



Donor

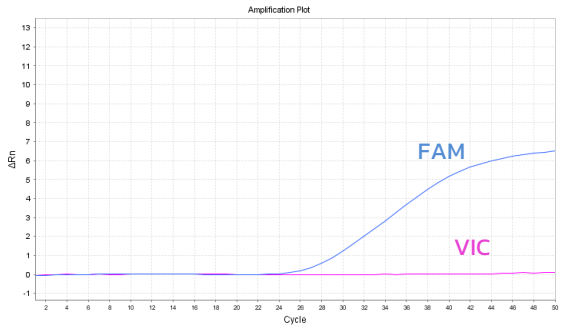


Figure 2. Results obtained on 7500 FAST Real-time PCR System for recipient and donor samples. Two genetic markers are multiplexed in each PCR reaction. The blue amplification curves represent the amplification signal in the FAM channel and the pink amplification curves represent the amplification signal in the VIC channel.

↘ Imegen®-Quimera Software, by Health in Code, S.L.

Health in Code, S.L. has designed and developed a user-friendly application that allows the user to create a patient database, as well as to register the results of the informative polymorphism screening, the quantifications of the informative polymorphism of the different samples of a patient's follow-up and the medical actions applied to the patient during their follow-up. Users can also view all the medical actions and the patient's course in a graph, and export the results.

There's a video tutorial on how the application works for users at the following link: youtu.be/K38cV3hacm8

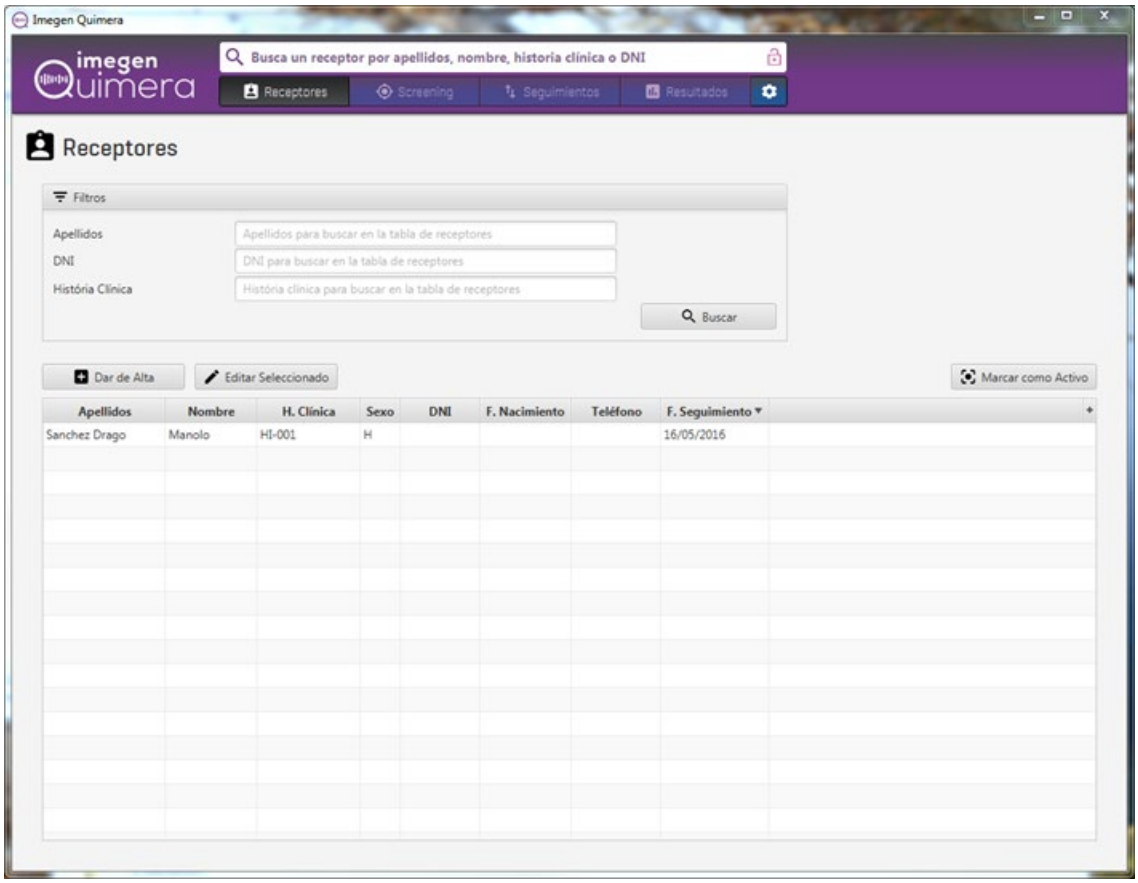


Figure 3. View of the patient follow-up app developed by Health in Code, S.L.

09 Troubleshooting

The table below presents the results that could be obtained from the analysis of different positive and negative controls and a genomic DNA sample in an assay, as well as their interpretation and the most probable cause for said result:

Control	C_T Polymorphism	Cause
Analyzed sample	Detected < 30	Expected result
	Detected > 30	PCR contamination with human DNA [1], sample concentration lower than specified in the protocol [2], or marker poorly represented in the sample [3].
	Not detected	Expected result
PCR negative control	Not detected	Expected result
	Detected	Contamination of PCR with human DNA [1]

Table 8. Possible results of controls and samples

(1) PCR contamination with DNA: contamination of PCR reactions may be due to a sample handling error, reagent contamination or environmental contamination. Thoroughly clean the laboratory where the PCR process was performed as well as the equipment. If necessary, use new aliquots of the reagents used in the PCR and repeat the assay.

(2) Inadequate sample concentration: late amplification of markers may be due to the DNA concentration used in the assay being lower than specified by the protocol. In this case, it is recommended to quantify the sample again by absorbance or fluorescence. If necessary, use new aliquots of the reagents used in the PCR and repeat the assay.

All the data from a sample must be analyzed jointly, because if the Cts from all the screening markers are delayed (Ct>30) this is probably due to an inadequate concentration of the sample and not due to contamination.

(3) Marker poorly represented in the sample: If Ct>30 is not a general result for all the screening markers, the markers with this result are not recommendable for future use in digital PCR follow-up of hematopoietic chimerism. In such cases, the Ct>30 result may be due to a low representation of this marker in the test sample. As a result, the user can consider these results inconclusive, despite the amplification curves being correct.

10 Limitations

10.1 | Equipment

Imegen® Quimera Screening Multiplex Plus has been validated using the following real-time PCR platforms:

- **7500 FAST Real-Time PCR System** (Thermo Fisher Scientific)
- **StepOne Plus Real-Time PCR System** (Thermo Fisher Scientific)
- **LightCycler 480** (Roche)

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

10.2 | Reagents

Imegen® Quimera Screening Multiplex Plus has been validated using the following Hot Start polymerase:

- **PCR Hot Start Master Mix (TaqMan™ Environmental Master Mix 2.0, Thermo Fisher Scientific)**

It is advised to use the PCR reagents recommended by the product manufacturer. If in 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.

11 Performance characteristics

11.1 | Validation samples

The Imegen® Quimera Screening Multiplex Plus kit is designed for the analysis of genomic DNA (gDNA). The qPCR systems for each marker in the kit have been fine-tuned with synthetic DNA (plasmid) samples.

In addition, it has used:

- a cohort of 33 real samples and in which there is at least one positive sample for each marker. The positive samples used in the validation come from the Biobank of Health in Code, S.L. and present the polymorphism in homozygosis or heterozygosis.
- 26 reference samples, obtained from the NIGMS Human Genetic Cell Repository of the Coriell Institute for Medical Research, harboring one of the polymorphisms of interest.

For a sample to be considered positive, a threshold cycle or Ct <30 has been established, provided that the instructions for sample preparation, type and starting sample concentration recommended in this manual have been followed.

11.2 | Sensitivity and analytical specificity

After fine-tuning the systems with specific plasmids for each marker, the evaluation of analytical sensitivity and specificity for all markers was carried out with the real and reference samples selected.

Initially, a positive and negative sample was chosen for each marker. These samples are first checked with the systems designed without multiplexing and then multiplexed in pairs. Figure 4 shows an example of multiplexed and individual PCRs of the 28I/23I markers, showing that there is no difference in PCR efficiency. All multiplexed systems matched the results of the individually evaluated systems.

Finally, sensitivity and specificity studies were extended with the rest of the real and reference samples.

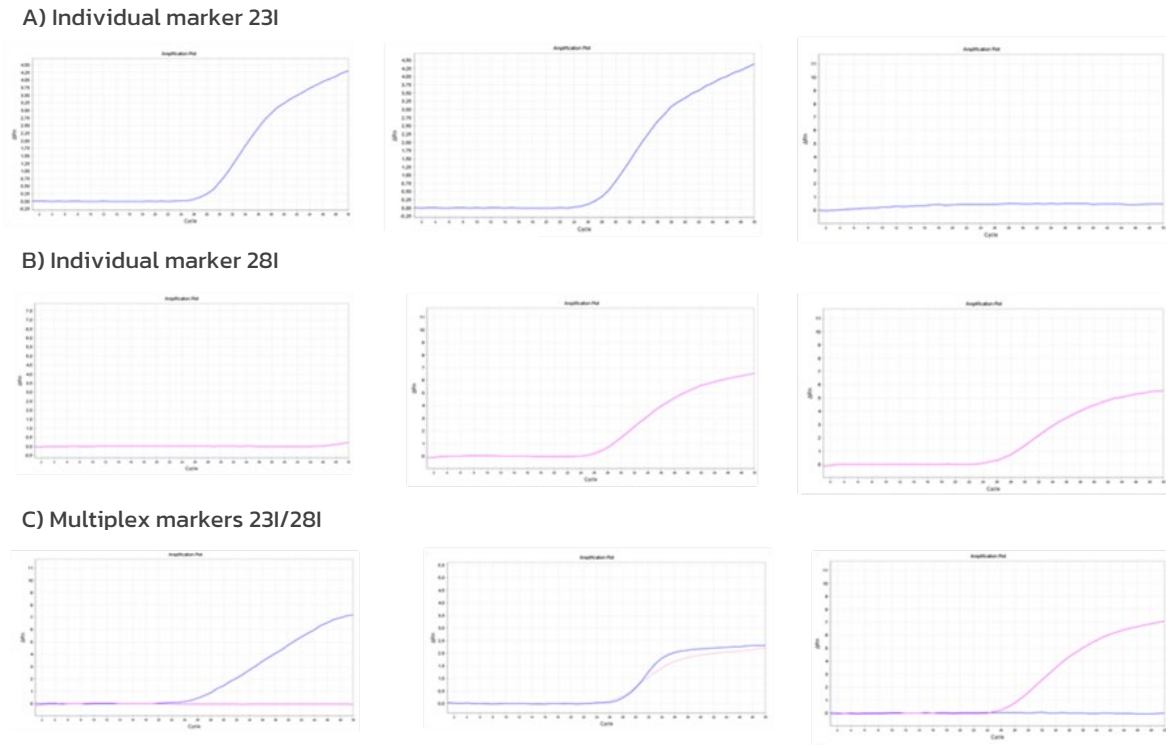


Figure 4. Results of the analytical specificity for markers 23I/28I separately and in a multiplex PCR system.

The results obtained determine that:

- the multiplex PCR systems show the same efficiency and specificity as for the 16 markers evaluated individually.
- the systems correctly identify true positives and true negatives, resulting in 100% sensitivity and specificity.

In all cases the systems designed have correctly determined the genotype of the samples.

11.3 | Limit of detection (LOD)

To study the LOD of the Imegen® Quimera Screening Multiplex Plus kit, the performance of the qPCR assay for each marker was evaluated by varying the starting gDNA concentration. For this purpose, a heterozygous reference sample was selected for the biomarker under analysis and decreasing amounts of gDNA were used, including the optimal amount recommended for the assay (50 ng, 5 ng and 1 ng). The dilutions obtained were checked with the DNAds Qubit™ (Thermo Fisher Scientific). Then, three replicates of each concentration were made and evaluated in the 7500 FAST and StepOne kits (Thermo Fisher Scientific).

In all cases and concentrations tested, the established coefficient of variation criterion (CV < 25%) was met. Therefore, the detection limit set is 1 ng total genomic DNA for samples with the polymorphisms in hetero- or homozygosity.

11.4 | Repeatability and reproducibility

The repeatability of the Imegen® Quimera Screening Multiplex Plus kit was evaluated for all markers by analyzing 3 replicates of a positive sample for the marker being evaluated, in the same assay, both with the multiplexed and individual qPCR system. Then, the CV between replicates of the same marker was calculated, which in all cases was less than 25% (established acceptance criterion).

To evaluate the reproducibility of each marker pair, three assays were performed on different days by different operators for the two validated kits (Applied Biosystems™ 7500 Fast as in the StepOnePlus™) with the characteristics indicated below:

- ASSAY 1: a positive sample is used for the two markers.
- ASSAY 2: a positive sample for one of the two markers is used.
- ASSAY 3: a positive sample is used for one of the two markers.

Table 9 shows the results obtained to determine the reproducibility of the kit. The results obtained make it possible to establish adequate precision for the Imegen® Quimeras Screening Multiplex Plus kit by obtaining a coefficient of variation of less than 25% between replicates and 100% concordance in the reproducibility experiments.

Mix	Marker	ASSAY 1		ASSAY 2		ASSAY 3		Concordance
		7500 FAST	StepOne Plus	7500 FAST	StepOne Plus	7500 FAST	StepOne Plus	
1	Q116-6I	24.7	27.05	24.52	27.05	N.D.	N.D.	100%
	Q116-3I	24.02	28.44	N.D.	N.D.	22.36	23.27	
2	Q116-7I	23.48	24.52	22.03	23.42	N.D.	N.D.	
	Q116-12D	23.65	24.57	N.D.	N.D.	23.88	22.85	
3	Q116-11I	23.37	24.12	22.37	22.76	N.D.	N.D.	
	Q116-5I	25.32	24.08	N.D.	N.D.	23.94	24.51	
4	Q116-4I	22.84	23.48	22.81	23.06	N.D.	N.D.	
	Q116-10I	24.47	24.48	N.D.	N.D.	24.50	24.96	
5	Q116-23I	22.85	23.45	23.15	24.06	N.D.	N.D.	
	Q116-28I	24.68	24.95	N.D.	N.D.	24.62	22.58	
6	Q116-32I	27.36	28.37	N.D.	N.D.	23.93	24.53	
	Q116-31I	29.11	29.96	27.8	26.52	N.D.	N.D.	
7	Q116-30D	24.64	25.75	N.D.	N.D.	29.94	30.04	
	Q116-29D	24.87	25.84	24.62	25.48	N.D.	N.D.	
8	Q116-27D	24.31	28.23	N.D.	N.D.	24.94	26.39	
	Q116-24I	28.66	31.34	27.73	30.17	N.D.	N.D.	

Table 9. Reproducibility test results.

N.D.: not detected: N.D. results correspond to negative samples for the marker with Cts > 30 or "Indeterminate".

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

tech.support@healthincode.com

+34 963 212 340

healthincode