Instructions for Use

Imegen™ Quimera qPCR

REF **IMG-116**

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Rev. 7. 11/12/2020

Imegen 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 diagnostics. The user of the product is responsible for validating the usefulness of the protocol proposed by Imegen. Imegen does not offer any other warranty, express or implied, which extend beyond the proper functioning of the components of this kit. Imegen 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.

Imegen 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 marketed by Imegen are subjected to rigorous quality control. **Imegen-Quimera Screening Multiplex Plus kit** has passed all internal validation tests, ensuring the reliability and reproducibility of each test.

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

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Amendments to the Instructions for Use (IFU)	
Version 05	Content review
Version 06	Correction of the reaction quantities in section 7.2
Version 07	Content review and adition of information regarding the new commercial kit IMG-116-74

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1. General information

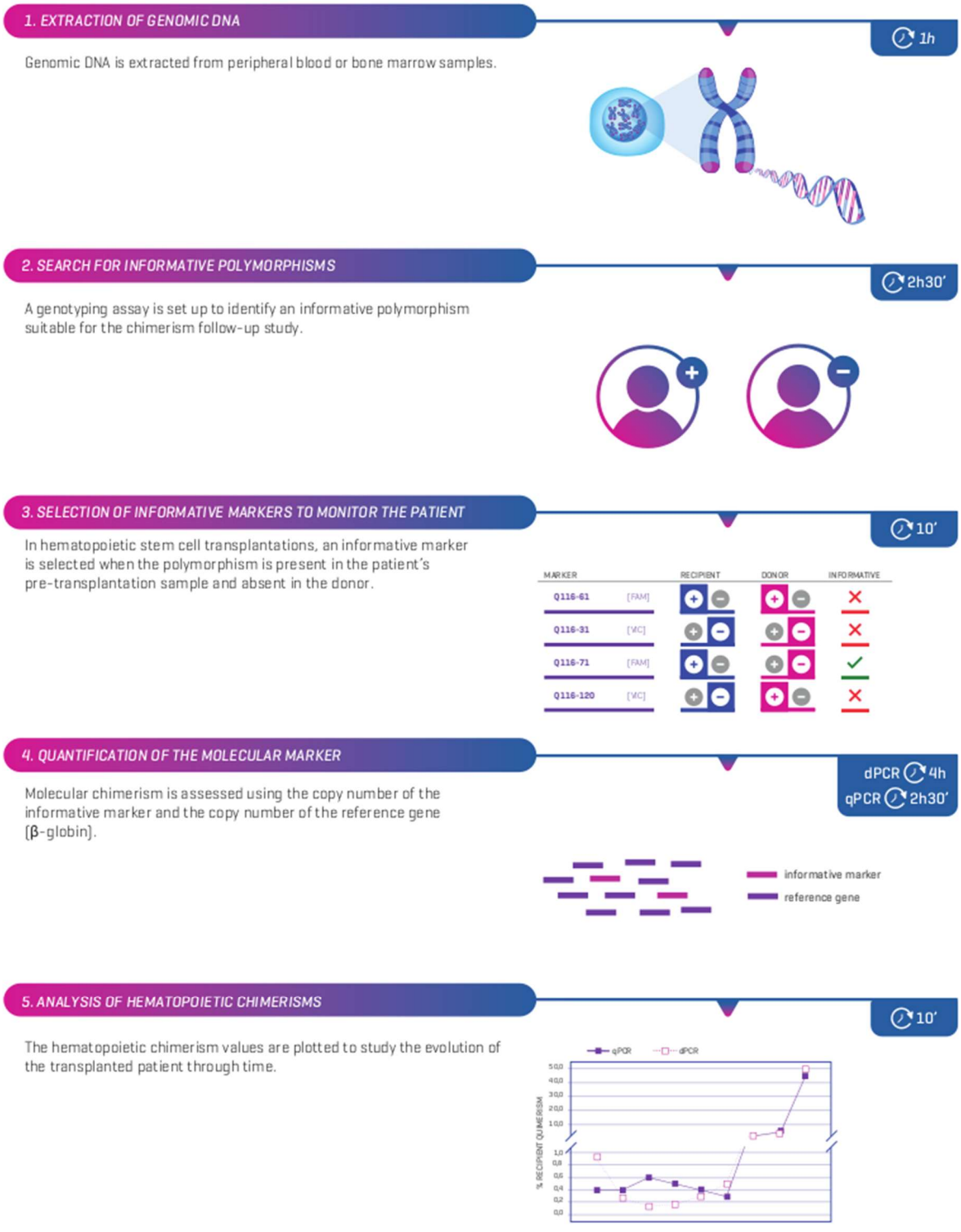
Molecular chimerism analysis has become a well-established method to monitor the transplant evolution after allogeneic stem-cell transplantation. It provides precise and valuable information that allows the clinician to understand the evolution of the patient as a guide to determine the most suitable course of treatment subsequently to solid organ transplantation or hematopoietic stem cell transplantation. Such an approach would be helpful not only in determining the risk of relapse, rejection or graft-vs-host disease, but in assessing the response to diverse treatment modalities.

The real-time PCR kits on the **Imegen-Quimera** family have been developed in collaboration with the Regional Hospital Carlos Haya in Malaga included in the Andalusian Health Service (SAS). The result of this agreement, Imegen has an **exclusive worldwide** license on the know-how of products for manufacturing and commercial exploitation of them.

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.

Chimerism analysis workflow:



2. Intended use

Imegen-Quimera enables the relative quantification of haematopoietic chimerisms in the post-transplantation period by real time PCR. This assay is based on the previous screening for informative polymorphisms, including INDELs (insertion or deletion) markers or null alleles which once selected can be used to quantify the chimerisms as the relative amount of informative marker to the total amount of genomic DNA, using a reference gene (*β-globin*).

In the first place, it is essential to select the informative polymorphisms for each transplantation case. For this, Imegen has validated the screening references **Imegen-Quimera Screening Multiplex I** (ref. IMG-116-24) and **imegen-Quimera Screening Multiplex II** (IMG-116-74. In bone marrow transplant is considered that a polymorphism is informative when it is present in the transplanted recipient and not in the donor.

Once an informative marker has been selected, it can be used to monitoring the transplanted patient. A total of 34 polymorphic markers are available for molecular chimerisms analysis, as follows:

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
Q116-12I	X		IMG-116-12
Q116-4D		X	IMG-116-13
Q116-5D		X	IMG-116-14
Q116-10D		X	IMG-116-17
RhD	X		IMG-116-18
Q116-20I	X		IMG-116-20
Q116-12D		X	IMG-116-21
Q116-23I	X		IMG-116-23
Q116-33I	X		IMG-116-16
Q116-37I	X		IMG-116-75

Markers	Insertion (Allele +)	Deletion (Allele -)	Kit reference
Q116-38I	X		IMG-116-76
Q116-44I	X		IMG-116-77
Q116-43I	X		IMG-116-78
Q116-49I	X		IMG-116-79
Q116-39I	X		IMG-116-80
Q116-50I	X		IMG-116-70
Q116-46I	X		IMG-116-66
Q116-47I	X		IMG-116-81
Q116-32I	X		IMG-116-82
Q116-31I	X		IMG-116-83
Q116-30D		X	IMG-116-84
Q116-29D		X	IMG-116-73
Q116-27D		X	IMG-116-85
Q116-24I	X		IMG-116-87

Table 1. Imegen-Quimera references for qPCR analyses

These instructions for use are suitable for the analysis of any of the 34 markers included in Table 1, since they work optimally under the same PCR conditions. Thus, this technique allows a quick and effective analysis of multiple polymorphisms simultaneously.

The **Imegen-Quimera qPCR** kit is designed for research use only and it is aimed at professionals in the Molecular Biology sector.

3. Technical characteristics

Imegen-Quimera qPCR consists in a real time PCR assay aimed at quantifying the copy number of one informative marker to haematopoietic chimerisms monitoring. It employs a combination of specific oligonucleotides and fluorescent hydrolysis probes to quantify the relative amount of the informative marker relative to a reference gene, β-globin.

The chimerism quantitative analysis implements a relative calculation system implemented by the method established by Pfafl that takes into account the efficiency of the amplification system and the values of "cycle threshold" (Ct) obtained for an endogenous control or calibrator, included in the kit as a positive control. From these values, the system allows the calculation of chimerism in each test. Likewise, Imegen has developed a calculation tool to facilitate the analysis of chimerism using real-time PCR.

Imegen-Quimera qPCR has been validated with genomic DNA (gDNA) extracted from peripheral blood and bone marrow samples from patients that have experienced an allogeneic stem cell transplantation. The limit of quantification (LOQ) has been established at 0.1%, whereas the limit of detection (LOD) is established to be 0.01% when genomic DNA samples are used.

This product meets the quality requirements established by ISO 9001, not only the materials used in its manufacturing process but the final marketed product as well.

4. Warnings and precautionary statements

1. Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.

2. Do not pipette by mouth.

3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

4. You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.

5. 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.

6. In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.

7. The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to Imegen.

8. 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.

9. Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.

10. This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.

11. 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 Imegen.

12. The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Imegen, considering them equivalent to those provided in the Kit.

5. Contents and storage conditions

The kit contains lyophilised reagents to perform 25 qPCR reactions:

- *Polymorphism* Master Mix*:* Oligonucleotides and hydrolysis probe for the amplification of the target polymorphism. The probe is labelled with FAM™ fluorochrome.

- β-globin Master Mix: Oligonucleotides and hydrolysis probe for the amplification of the reference gen (β-globin) that functions as an active reference for the relative quantification of the chimerism. The probe is labelled with FAM™ fluorochrome.

- Positive control: It is a standard that will act as a calibrator and will allow the relative quantification of the markers, as well as the control of the evolution of the transplant, without the need of disposing and store a large amount of the recipient's sample before the transplant. This standard is a synthetic plasmid that contains each homozygous polymorphism. Therefore, and although it is not the main objective, it also provides information on whether the polymorphism is in the sample in homozygosis (result = expected percentage) or in heterozygosis (result = half of the expected percentage).

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

Table 2. Imegen-Quimera kit for qPCR contents and storage temperature

*Reagents are lyophilized. Once the reagents are rehydrated, the vials should be stored at -20°C.

6. Equipment and materials required but not supplied

Equipment:

- Real time PCR Thermal Cycler (FAM™ Channel)
- Micropipettes (10 µL, 20 µL and 200 µL)
- Vortex

Reagents:

- Nuclease-free water

Materials:

- Disposable micropipette filter tips (10 µL, 20 µL and 200 µL)
- 1.5 mL sterile tubes
- Optical 96-well reaction plates or 0,2 mL optical tubes
- Optical adhesive films for 96-well plates or optical covers for 0.2 mL tubes
- Powder-free latex gloves

6.1 Related Kits

A previous step to the informative marker quantification consists in determining the informativity of the possible polymorphisms by real time PCR; for this, Imegen has developed the **Imegen-Quimera Screening Multiplex I** (Ref. IMG-116-24) and **Imegen-Quimera Screening Multiplex II** (Ref. IMG-116-74).

7. Assay protocol

7.1 Preparation of the PCR reagents

All reagents included in the kit are lyophilised. The first step before using any of our kits consist of reagents rehydration by adding the amount of nuclease-free water, showed in the following table. In order to facilitate resuspension of each component, we recommend stirring well and spinning the tubes and store them at 4ºC for one hour before their use.

Reagents	Rehydration volume
Polymorphism Master Mix	*80 µL of water/vial
ß-globina Master Mix	*80 µL of water/vial
Positive control	*100 µL of water/vial

Table 3. Kit components rehydration volume

If these reagents will not be used after rehydration, we recommend store them at -20 º C.

7.2 Setup of the PCR assay

The assay should include the following reactions:

- Each sample reactions per duplicate.
- Positive control reactions per duplicate.
- Negative control reaction (reaction which contains water instead of DNA in order to check if there is contamination in the PCR master mixes).

Relative quantification with Imegen-Quimera kits requires two different PCR mixes preparation: one to analyse the reference gene (β-globin) and other to analyse the informative polymorphism. Each PCR master mix contains:

- β-globin Master Mix or polymorphism Master Mix
- 2x PCR Master Mix

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

1. Thaw the following reagents for the analysis:
    a. Genomic DNA samples diluted at the optimal concentration (25 ng/µL).
    b. Rehydrated polymorphism Master Mix
    c. Rehydrated β-globin Master Mix
    d. Nuclease-free water for the negative controls
    e. Positive control
    f. Real time PCR Master Mix (2X), not included in the kit.
2. Vortex and spin each reagent and keep them cold.
3. Prepare two PCR master mixes by adding the specified amount of each reagent into a 1.5 mL tubes. To estimate the amount of necessary reagents, we recommend make calculations taking into account the number of samples to be simultaneously analysed, and then considering one more reaction, or increase a 10% the volume of each reagent.

Reagents	Amount per reaction
Polymorphism Master Mix	3 µL
2X PCR Master Mix	5 µL

Reagents	Amount per reaction
β-globin Master Mix	3 µL
2X PCR Master Mix	5 µL

4. Vortex each PCR Master Mix tube and dispense 8 µL into the corresponding wells.
5. Add 2 µL of sample DNA at 100 ng/µL, of the positive control or of nuclease-free water (negative control) into the corresponding wells.

Little concentrated samples

Sometimes, the first post-transplantation sample collection results in a sample with a low concentration of DNA. In this case it is recommended to prepare amplification reactions with the following amounts of reagents:

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

Table 4. Amount of reagents necessaries for little concentrated samples reaction

7.3 Setup of the real time PCR program

The following instructions must be followed in order to setup the amplification program:

<u>7500 Fast or StepOne Real-Time PCR system (Thermo Scientific)</u>

- Experiment type: *Quantitation- Standard curve*
- Ramp rate: *standard*
- Reaction volume: 10 µL
- Baseline reference ROX™: include
- TaqMan® probes fluorophores:

Probe	Receptor	*Quencher*
ß-globin	FAM™	TAMRA*
Informative polymorphism	FAM™	TAMRA* (MGB for the IMG-116-23 reference)

Tabla 5. Probes information

** In StepOne this field should be indicated as "None".*

- Optimal program:

Fields	Step 1 Enzymatic activation	Step 2 PCR	
Cycles	1 Initial cycle	50 cycles	
		Denaturation	Primers binding/ Extension
Temperature	95°C	95°C	58°C
Time	10 minutes	15 seconds	1 minute*

Table 6. Optimal PCR program for 7500 FAST or StepOne

**Fluorescence detection*

480 Lightcycler (Roche)

- Optimal program:

Fields	Step 1 Enzymatic activation	Step 2 PCR			Step 3
Cycles	1 Initial cycle	50 cycles			1 Final cycle
		Denaturation	Primers binding	Extension	
Temperature	95ºC	95ºC	58ºC	72ºC	40ºC
Time	10 minutes	5 seconds	10 seconds	15 seconds *	20 seconds

Table 7. 480 Lightcycler PCR optimal program

**Fluorescence detection*

8. Results analysis

In order to perform the results analysis it is recommended following the instructions:

- Check that there is not amplification in negative controls. If there is amplification, it should be repeated the analysis to rule out an accidental contamination.

- Check that the reference gene (β-globin) is detected in a PCR cycle less than 26 in both, in the standard and in the samples. Amplifications signals in Ct above this indicates that the amount of DNA in the sample is not enough, resulting in a decrease in the sensitivity of performed analysis.

- For the relative quantification of hematopoietic chimera it has developed specific software, available in www.imegen.es/Quimera. This software will allow you to get the result once you enter the following parameters:

  a. Select the polymorphism used in the analysis.
  b. Indicate the Ct of the standard or calibrator for both, the β-globin and the informative polymorphism master mixes.
  c. Indicate Ct samples for both, the β-globin and the polymorphism master mixes.

- If you want to perform calculations manually, use the following formula:

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

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

Imegen-Quimera Software (Application for the patient follow-up)

Imegen has designed and developed an easy-to-use application that allows the user to create a patient database, as well as recording 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 his follow-up. In addition, the user can visualize all the medical actions and the evolution of the patient on a graph, in addition to exporting the results.

We put at your disposal a video tutorial of the use of our application Imegen-Chimera in the following link: https://www.youtube.com/watch?v=qvgtP83MiRg

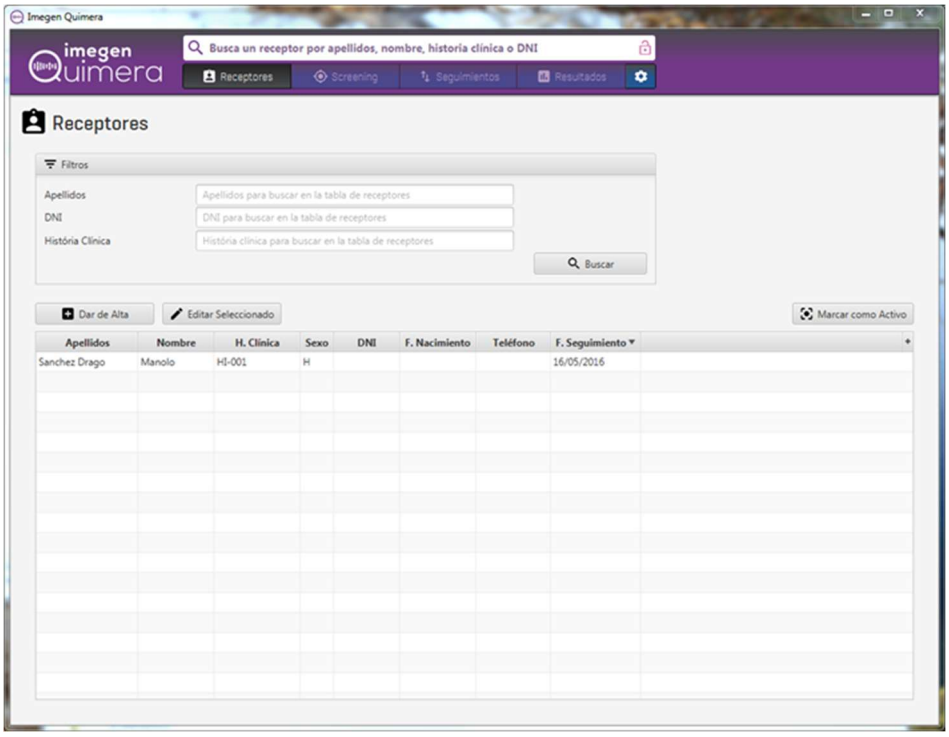


Image 1: View of the patient follow-up application developed by Imegen

9. Troubleshooting

The table below shows graphically the results that could be obtained from the analysis of the different controls and a sample in one trial, as well as their interpretation:

Control	Polymorphism	ß-Globin	Cause
DNA sample	+	+	Expected result
	-	+	Expected result if an informative marker is being analysed
	-	-	Fail to amplify the sample[1]
Positive control	+	+	Expected result
	-	-	Fail in the PCR setup[2]
Negative control	-	-	Expected result
	+	+	Contamination with human DNA or with the positive control[3]

Table 8. Interpretation of the possible results obtained with Imegen-Quimera qPCR kits

[1] **Fail to amplify the sample:** An error to amplify might suggest the quantity or the quality of the sample is compromised. In this situation, a second analysis or new extraction of DNA would be recommended before an interpretation of the results is made.

[2] **Fail in the PCR setup**: An error in the amplification might be due to a technical issue during the configuration of the PCR setup. Check the amplification program and the setup of the fluorescence detection.

[3] **Contamination with human DNA or with the positive control:** PCR contamination might be caused by an inappropriate handling of the sample, the use of contaminated reagents or caused by an environmental contamination. To solve this issue, a thorough cleanse of the laboratory where the PCRs are prepared, including the equipment and material used is recommended. If necessary, use fresh aliquots of the PCR reagents and prepare last, the PCR reactions containing the positive controls in order to avoid any cross contamination.

Other problems that may appear are:

Resulting amplification curves are not ROX normalized

Check that ROX fluorochrome has been configured as a passive reference in order to normalize well to well pipetting or equipment variations.

Poor reproducibility between replicates

- Pipetting errors
  Proposed Solution: Avoid variations between pipetting by using gloves, filter tips and pipettes calibrated properly.
- Variations due to PCR Master mixes
  Proposed solution: Prepare enough PCR master mix in order to prepare all PCR reactions and ensure that the master mix is well homogenized before its use (we recommend using vortex).
- Target DNA concentration close to the detection limit: Reproducibility between replicates may be compromised if the target DNA has a concentration near the detection limit.

Poor signal or not detected

- One or more components were not added to the reaction or were not in the reaction at an appropriate concentration.
  Proposed solution: Prepare againg the master mixes and repeat the assay if it is necessary.
- Fluorescence reading failure

  Proposed solution: Make sure that the fluorescence signal is captured in appropriated step and channel. Check that probes have been configured in the amplification program, associated with the appropriate fluorochrome and ROX/fluorescein levels are correct.
- Inhibidors presence
  Proposed Solution: Sometimes, some inhibitors are dragged from the extraction process along with DNA and they make difficult or impossible the amplification reaction. If this occurs, it can be purified or diluted the DNA to eliminate or minimize the presence of inhibitors in the reaction.
- Suboptimal conditions in the thermocycler
  Proposed Solution: Check the real time PCR amplification conditions are appropriate.
- Baseline and/or Threshold problems
  Proposed solution: Establish Baseline and/or Threshold values manually and above the baseline values in order to obtain accurate Cp/Ct values.

10. Limitations

10.1 Equipment

Imegen-Quimera family kits have been validated using the following real-time PCR platforms:

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

If you use another brand or model of thermal cycler, maybe you may need to adjust the amplification program. Please contact our technical service for any questions or clarification.

10.2 Reagents

It is recommended to use the PCR reagents recommended by the thermal cycler supplier to be used for real-time PCR assays. If in doubt, please contact our technical service.

It is recommended to use a HotStart Polymerase (Not Fast enzyme).

10.3 Product stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied as specified on Section 5 (Contents and Storage Conditions) from the reception of the kit until the expiry date assigned to each production batch.