Instructions for use

Imegen® Gilbert Plus

Ref. IMG-288

CE IVD

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

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

Our products are intended for *in vitro* diagnostic use. Health in Code S.L. provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code's sole obligation, in relation to the aforementioned guarantees, shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship prove to be defective.

Health in Code S.L. is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user as a result of use of the product by the buyer or user.

All Health in Code S.L. products undergo strict quality control. Imegen® Gilbert Plus has passed all internal validation tests, thereby ensuring the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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Instructions for Use (IFU) modifications		
Version 05	NOV 2022	Change in manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 04	MAY 2022	Change in manufacturer identification: from Imegen S.L. to Health in Code S.L.
Version 03	FEB 2020	Change in the PCR amplification program and positive control volume adjustment. The change applies from batch 28820C007 onwards.
Version 02	NOV 2018	Document update for the product's CE-IVD.

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01 General information

Gilbert syndrome is an autosomal recessive hereditary disease that manifests as high levels of unconjugated or indirect bilirubin in the blood (hyperbilirubinemia) and is caused by partial deficiency of the glucuronyltransferase (UGT) enzyme, which is encoded by the UGT1A1 gene and is responsible for the elimination of bilirubin from the liver. This deficiency is caused by dinucleotide expansions (TAx) in the TATA-box, which promotes transcription of the UGT1A1 gene, located in the chromosomal region 2q37.1. The presence of the $A(TA)_7TAA$, $A(TA)_5TAA$, and $A(TA)_8TAA$ alleles in the regulatory region of the gene alters the normal transcription of the bilirubin-UGT enzyme, resulting in Gilbert syndrome.

Though it may cause mild jaundice as a clinical manifestation, Gilbert syndrome generally does not cause symptoms.

References

- *Molecular pathogenesis of Gilbert's syndrome: Decreased TATA-binding protein binding affinity of UGT1A1 gene promoter.*
  Request PDF Available from:
  https://www.researchgate.net/publication/6334763_Molecular_pathogenesis_of_Gilbert's_syndrome_Decreased_TATA-binding_protein_binding_affinity_of_UGT1A1_gene_promoter

02 Intended use

The Imegen® Gilbert Plus kit is a robust and specific test developed for the detection of the mutant alleles—A(TA)$_5$TAA, A(TA)$_7$TAA, and A(TA)$_8$TAA—as well as the normal of the promoter of the UGT1A1 gene—A(TA)$_6$TAA—via PCR and fragment analysis.

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

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

03 Technical characteristics

This kit has been validated using reference samples provided by the *Coriell Institute for Medical Research* and samples previously analyzed by Health in Code S.L.'s *Medical Genetics* team, and it specifically detects the alleles for which it has been designed.

The material required for this test is genomic DNA from mainly peripheral blood. The total necessary amount of DNA is 50 ng.

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

- Genetic testing kits
- Software for the bioinformatics analysis of genetic data

04 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory conduct an internal validation when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code S.L.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code S.L. but are considered by the user equivalent to those provided in the kit.

05 Content and storage conditions of the kit

This kit contains enough reagents to perform 48 reactions. The reagents included in this kit are as follows:

- **Gilbert Plus Master Mix**: Contains the oligonucleotides necessary for the amplification of the target region of the kit.
- **General Master Mix I**: PCR master mix with the necessary amounts of enzyme, nucleotides, $MgCl_2$, and buffer to prepare the reactions.
- **Positive Control**: Mix of gDNA containing the normal allele, $A(TA)_6TAA$, and the following mutant alleles: $A(TA)_5TAA$, $A(TA)_7TAA$, and $A(TA)_8TAA$.

Reagents	Color indicator	Quantity	Conservation
Gilbert Plus Master Mix	Green disc	2 x 180 µl	-20°C
General Master Mix	White disc	600 µl	-20°C
Positive Control	Orange cap	60 µl	-20°C

Table 1. Components of the Imegen® Gilbert Plus

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
- Sequencer

Reagents:

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

Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 1000µL)
- Sterile 1.5 mL tubes
- 0.2-ml 96-well plates or tubes
- Sealing film for 96-well plates
- Latex gloves

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

07 Assay protocol

07.1 | Preparation of amplification reactions

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

01 Thaw all kit reagents and RNA samples at room temperature and keep on ice once thawed. Shake each reagent on a vortex mixer and keep cold.

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

Reagent	Volume per reaction
Gilbert Plus Master Mix	7.5 µL
General Master Mix I	12.5 µL

NOTE: To estimate the necessary amount of reagents, we recommend that the calculations be made based on the number of samples and increasing the volume of each reagent by 10%.

03 Mix on a vortex mixer and spin the PCR mix. Dispense 20 µL to the corresponding 0.2-mL tubes.

04 Once the PCR mix has been dispensed, add the following amounts to the corresponding tubes:

- 5 µL of genomic DNA from the sample (10 ng/µL).
- 5 µL of positive control.
- 5 µL of nuclease-free water (negative control).

NOTE: It is convenient to include a negative control in each round of amplification to verify the absence of contamination of the reagents, and a positive control to verify the size of the alleles.

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

Fields	Phase 1	Phase 2			Phase 3	
No. of cycles	1 initial cycle Enzyme activation	35 cycles			1 final cycle Final extension and conservation	
		Denaturation	Primer binding	Extension		
Temperature	95°C	95 °C	62 °C	72°C	72°C	4°C
Time	10 minutes	30 seconds	30 seconds	45 seconds	10 minutes	∞

Table 2. Optimal PCR program for T3 by Biometra and SimpliAmp Thermal Cycler and GENEAMP® PCR System 2720 by 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 | Fragment analysis preparation

Using the PCR products, prepare the plate for fragment analysis as follows:

01 In a 1.5-mL tube, add the following reagents:

Reagent	Volume per reaction
Formamide	18 µL
GeneScan™ 500 LIZ marker	0.5 µL

We recommend either including one extra reaction in the calculations or increasing the volume of each reagent by 10%.

NOTE: The volume of the size marker can be increased or decreased to adjust the intensity of the peaks.

02 Dispense 18.5 µL of the mix in each well.

03 Add 1 µL of DNA obtained by PCR.

NOTE: The volume of the sample can be increased or decreased (by diluting the samples) to adjust the intensity of the peaks.

05 Cover and spin the plate and denature in a thermal cycler for 5 minutes at 98 °C.

06 Store the plate at 4 °C until you are ready to load it into the sequencer.

07.3 | Capillary electrophoresis

Once the fragment plate is prepared, the reactions should be subjected to capillary electrophoresis. Depending on the type of sequencer used, electrophoresis conditions recommended by the manufacturer shall be used.

To set the conditions for capillary electrophoresis, it should be borne in mind that the amplification range varies approximately between 315-330 bp, that 6-FAM-labelled primers are employed, and the molecular weight pattern is marked by *GeneScan™ 500 LIZ*.

The following image shows the optimal parameters for the *3730xl DNA Analyzer* sequencer (ThermoFisher Scientific) when the *POP-7™* polymer is used.

Run Module Settings

Name	Value	Range
Oven_Temperature	63	18...70 DegC
Buffer_Temperature	35	30...35 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.6	0...15 kV
Injection_Time	15	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	15.0	0...15 kV
Voltage_Number_Of_Steps	10	0...100 Steps
Voltage_Step_Interval	20	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	1	1...1800 sec
Data_Delay	500	1...1800 sec
Run_Time	1600	300...14000 sec

Ok Cancel

Figure 1. Optimal parameters for the 3730xl DNA sequencer

Detection intensity may vary between different instruments, depending on the model, the state of the optical system, and the injection time and voltage. Therefore, in some cases it may be necessary to dilute the samples before capillary electrophoresis is carried out.

08 Analysis of results

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

- Specific software and the .fsa file resulting from the capillary electrophoresis must be used to analyze the samples.
- Verify that there are no peaks of 315–330 base pairs in the electropherogram of the negative PCR control. If amplification is detected, it is recommended to repeat the assay to rule out accidental contamination.
- Sample analysis:
  - The size of the fragments may vary between different laboratories, depending on the reagents and sequencers used for fragment analysis.
  - To know which allele has a problem sample, it should be compared with the positive control, which contains the three mutant alleles ($A(TA)_5TAA$, $A(TA)_7TAA$ y $A(TA)_8TAA$) as well as the normal allele, $A(TA)_6TAA$ (Figure 2).

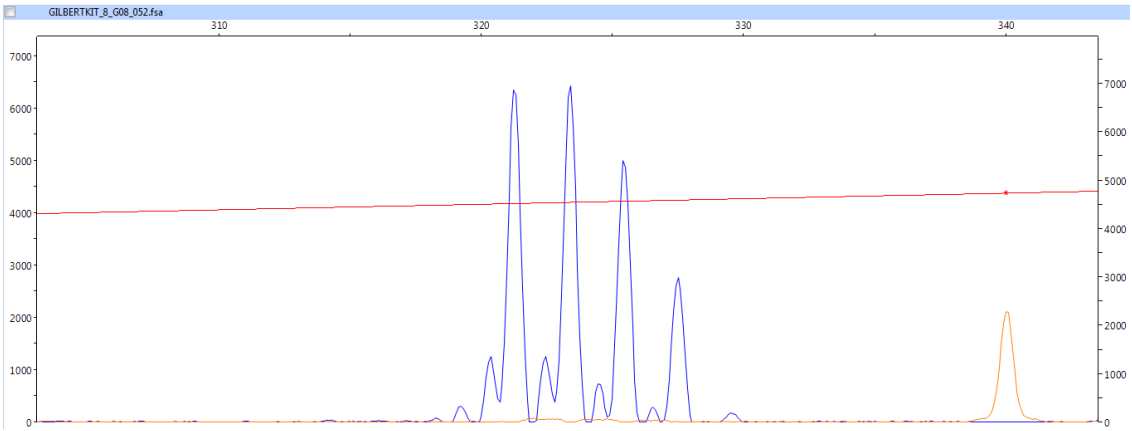

Figure 2. Result obtained from the positive control. From left to right the peaks correspond to the $A(TA)_5TAA$, $A(TA)_6TAA$, $A(TA)_7TAA$), and $A(TA)_8TAA$ alleles.

The following table outlines the correlations between allele and size:

Allele	Size (bp)
$A(TA)_5TAA$	321
$A(TA)_6TAA$	323
$A(TA)_7TAA$	325
$A(TA)_8TAA$	327

Table 3: Alleles and size of the positive control

The electropherogram can be used for allelic discrimination and clearly establishes the genotype of the sample, determining the alleles present in the sample based on the size of the peaks and, depending on the number of peaks, whether it is homozygous or heterozygous for these alleles.

	$A(TA)_6TAA$	$A(TA)_7TAA$	$A(TA)_5TAA$	$A(TA)_8TAA$
$A(TA)_6TAA$	Homozygous $(TA)_6$	Heterozygous $(TA)_6/(TA)_7$	Heterozygous $(TA)_6/(TA)_5$	Heterozygous $(TA)_6/(TA)_8$
$A(TA)_7TAA$	Heterozygous $(TA)_6/(TA)_7$	Homozygous $(TA)_7$	Heterozygous $(TA)_7/(TA)_5$	Heterozygous $(TA)_7/(TA)_8$
$A(TA)_5TAA$	Heterozygous $(TA)_6/(TA)_5$	Heterozygous $(TA)_7/(TA)_5$	Homozygous $(TA)_5$	Heterozygous $(TA)_5/(TA)_8$
$A(TA)_8TAA$	Heterozygous $(TA)_6/(TA)_8$	Heterozygous $(TA)_7/(TA)_8$	Heterozygous $(TA)_5/(TA)_8$	Homozygous $(TA)_8$

Table 4. Genotypes that can be identified by Imegen® Gilbert Plus

NOTE: In samples with homozygous genotypes, only one peak corresponding to the size of the allele that they present in homozygosity is observed.

09 Troubleshooting

The following table shows the results that could be obtained from the analyzed samples, the positive control, the size marker, and the negative control. If an unexpected result is obtained, the interpretation and the most likely reason for the result are given in the following table:

Issue	Analyzed samples	Positive control	Size marker	Negative control	Result / Interpretation
Weak or no fluorescence signal				√	Expected result
	√			√	Insufficient template DNA quantity and/or quality [1] Impure template DNA [2]
	√	√	√	√	Capillary electrophoresis failure [3] Denaturation failure [4]
	√	√		√	PCR failure [5]
Excessive intensity of the fluorescence signal	√				Excessive amount of DNA [6]
	√	√			
More peaks than expected appear	√	√		√	Contamination [7]
	√				
	√	√			Artifacts characteristic of expansions [8]

Table 5. Interpretation of the possible results of Imegen® Gilbert Plus

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

(2) Impure template DNA: High concentrations of salts or altered pH can inhibit PCR. If DNA dissolved in an elution buffer with a pH other than 8 or with high EDTA concentrations is used, the volume of DNA should not exceed 20% of the total reaction volume. Remnants of reagents used during the extraction may also affect the PCR reaction. Should that be the case, the DNA shall be cleaned or a new extraction shall be prepared.

(3) Failure in capillary electrophoresis: Verify that the equipment parameters are as specified and insert the samples again.

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

(5) PCR failure: Verify that the used PCR program is correct.

(6) Excessive amount of DNA: Make sure you are using the appropriate amount of DNA. If so, dilute the PCR product in sterile deionized water and prepare again for denaturation and analyze on the sequencer.

(7) Contamination: It can be produced by other template DNA or by previously amplified DNA. Cross contamination can lead to false positives and result in errors in the interpretation of the results. Use filter pipette tips and change gloves regularly.

(8) Artifacts characteristic of expansions: The amplification of expansions generates artifacts (peaks in the electropherogram) that appear as peaks that are less intense and smaller in size than the predominant peak.

10 Limitations

10.1 | Equipment

Imegen® Gilbert Plus 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 service.

Imegen® Gilbert Plus has been validated using the following sequencing platform:

- *3730xl DNA Analyzer* (ThermoFisher Scientific)

This kit can be used with polymers compatible with 6-Carboxyfluorescein (6-FAM) labeling. If a piece of equipment different from the one mentioned above is used, follow the protocol specifications of the platform..

10.2 | Reagents

Imegen® Gilbert Plus 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 instrument manufacturer: **ThermoFisher Scientific**.

Should you have any questions, please contact our technical support team.

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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