Instructions for use

Imegen® HFE

Ref. IMG-218

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 all its products are free of defects, both in relation to the materials used and the manufacturing process. This guarantee is valid until the expiration date, provided that the storage conditions set out in this manual are followed.

Our products are designed for *in vitro* diagnostic use. Health in Code S.L. makes no other express or implied guarantee, which 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 will be to replace the products or refund the purchase price thereof, 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® HFE kit has passed all internal validation tests, which guarantee the reliability and reproducibility of each manufactured batch.

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

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Modifications to the instructions for use (IUF)		
Version 09	DEC 2022	Updating of references in section 1. Modifications in sections 2, 3, 5 and 8.
Version 08	DEC 2022	Modification of the storage and shipping temperature of the GENERAL MASTER MIX reagent (Section 5).
Version 07	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 06	SEP 2022	Change of manufacturer's identification: from Imegen to HEALTH IN CODE, S.L.
Version 05	JAN 2022	Modification of the positive control in section 7. Assay protocol, Introduction of heterozygous controls in section 5. Contents and storage conditions. All changes are included from batch 21820C004 onwards.

index

01 General information

The *HFE* gene, located on chromosomal region 6p22.2, encodes a membrane protein (belonging to the HLA-A family of major histocompatibility system molecules), which is involved in the regulation of the hormone hepcidin, considered crucial in the regulation of iron absorption and kinetics in the body and which is expressed in organs such as the liver and small intestine at high levels.

The p. His63Asp, p.Ser65Cys and p.Cys282Tyr mutations in the *HFE* gene prevent the HFE protein from reaching the cell surface and therefore it cannot interact with hepcidin and transferrin receptors. This causes too much iron to be absorbed in the diet, which ends up accumulating in the organs, especially in the liver, generating free radicals, which cause organ damage: hemochromatosis.

References

- *Martha-Spyridoula Katsarou, et al. Hemochromatosis: Hereditary hemochromatosis and HFE gene. Elsevier, Vitamins and Hormones, 2019; Volume 110.*
- *Antonello Pietrangelo. Hereditary Hemochromatosis: Pathogenesis, Diagnosis, and Treatment. Gastroenterology, 2010; Volume 139, Issue 2.*
- *E. H. Hanson, G. Imperatore, W. Burke. HFE Gene and Hereditary Hemochromatosis: A HuGE Review, American Journal of Epidemiology, 2001; Volume 154, Issue 3.*
- *Paulo C. J. L. Santos. Molecular Diagnostic and Pathogenesis of Hereditary Hemochromatosis. Int. J. Mol. Sci. 2012, 13(2), 1497-1511*
- *https://www.omim.org/entry/613609*

02 Intended use

The Imegen® HFE kit uses a combination of oligonucleotides and fluorescent hydrolysis probes in a validated qualitative real-time PCR analysis to simultaneously detect variants in the *HFE* gene associated with hemochromatosis. Specifically, the assay can detect the following alleles:

- HFE63 [NM_000410.3:c.187C>G (p.His63Asp)]
- HFE65 [NM_000410.3:c.193A>T (p.Ser65Cys)]
- HFE282 [NM_000410.3:c.845G>A (p.Cys282Tyr)]

This genetic analysis detects the presence or absence of such genotypes in three multiplexed real-time PCR reactions, which include simultaneous amplification of the reference genotype and the alternative genotype.

Imegen® HFE is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

This kit, Imegen® HFE, has been validated on the following platforms:

- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Plus Real-Time PCR System* (ThermoFisher Scientific)

The following were used for the validation: medical genetic samples from Health in Code S.L., previously analyzed by NGS; reference samples from the Coriell Institute; and synthetic DNA with the sequence of interest (manufactured and certified by GenScript).

Technical specifications:

- Type of sample: Genomic DNA from peripheral blood.
- Recommended quantity of DNA: 150 ng of DNA.

The Imegen® HFE kit is compatible with real-time PCR platforms with FAM™ and VIC™ fluorescence channels.

Health in Code S.L. is certified according to **the standard UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems – Requirements for regulatory purposes** by the AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS (AEMPS, Spanish Agency for Medicinal Products and Medical Devices) for the design, development and production of *in vitro* diagnostic medical devices:

- Genetic analysis 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 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. It is recommended that 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.
- 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 responsible 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.
- The manufacturer is not responsible for the results obtained when the bioinformatics analysis is performed on an analysis platform other than **Client Site**.

05 Content and storage conditions of the kit

This kit contains sufficient reagents in order to make 48 determinations. The list of reagents included in the kit is as follows:

- *HFE282 Master Mix:* Specific oligonucleotides, FAM™ and VIC™–labeled probes, and nuclease–free water for the detection of wild–type and mutated alleles (c.845G>A).
- *HFE63 Master Mix:* Specific oligonucleotides, FAM™ and VIC™–labeled probes, and nuclease–free water for the detection of wild–type and mutated alleles (c.187C> G).
- *HFE65 Master Mix*: Specific oligonucleotides, FAM™ and VIC™–labeled probes, and nuclease–free water for the detection of wild–type and mutated alleles (c.193A> T).
- *General Master Mix*: PCR Master Mix with the nucleotides, $MgCl_2$, enzyme and buffer required to perform real–time PCR.
- *Positive control:* Heterozygous Coriell reference samples for each system.

Reagents	Color	Quantity	Storage
HFE282 Master Mix	Purple pad	2 x 180 µL	–20°C
HFE63 Master Mix	Green pad	2 x 180 µL	–20°C
HFE65 Master Mix	Blue pad	2 x 180 µL	–20°C
General Master Mix	White pad	2 x 900 µL	–20°C*
HFE282 C+	Purple cap	40 µL	–20°C
HFE63 C+	Green cap	40 µl	–20°C
HFE65 C+	Blue cap	40 µL	–20°C

Table 1. Imegen® HFE kit components

(*) General Master Mix: It is recommended to keep frozen until first use, protected from light, and stored between 2– 8°C after first use.

06 Equipment, reagents and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler (FAM and VIC channels)
- 10 µL, 20 µL and 200 µL micropipettes
- Vortex
- Centrifuge

Materials:

- Pipette tips with filter (10 µL, 20 µL, 200 µL)
- 1.5 mL sterile tubes
- Optical consumables compatible with the real-time PCR thermal cycler
- Latex gloves

Complementary kits

For the genotyping analysis of targets related to hematological diseases, and specifically to alterations in the coagulation process, Health in Code S.L. also offers the following kits:

- Imegen® Cambridge II (ref. IMG-199)
- Imegen® MTHFR (ref. IMG-212)
- Imegen® Factor II (ref. IMG-214)
- Imegen® Factor XII (ref. IMG-215)
- Imegen® MTHFRII (ref. IMG-216)
- Imegen® Factor V (ref. IMG-217)

All of them, together with the Imegen® HFE kit, have been validated using the same real-time PCR program, so they can be analyzed together.

07 Assay protocol

07.1 | Preparation of amplification reactions

In order to estimate the quantity of reagents required, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend adding one more reaction or increasing the volume of each reagent by 10% when making the calculations.

In order to carry out the qualitative analysis, it is recommended to prepare one amplification reaction per sample for each of the three systems analyzed (HFE282, HFE63 and HFE65) and to include a negative PCR control to rule out contamination of the reagents, and a positive control to ensure the proper operation of the amplification program.

> IMPORTANT: A positive and a negative control must be included in the assay if you wish to use the Auto-calling tool in the results analysis to obtain the genotyping of a sample automatically with the analysis software.

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

01 Thaw all kit reagents and DNA from the samples.

02 Vortex each of the reagents and keep cold.

03 Prepare the three PCR mixes in 1.5 mL tubes by adding the following reagents per sample:

	Quantity per reaction		
Reagents	**HFE282 mix**	**HFE63 mix**	**HFE65 mix**
General Master Mix	12.5 µL	12.5 µL	12.5 µL
HFE282 Master Mix	7.5 µL	-	-
HFE63 Master Mix	-	7.5 µL	-
HFE65 Master Mix	-	-	7.5 µL

04 Vortex and spin the PCR mix, then dispense 20 µL into the corresponding wells of the optical consumables.

05 Add 5 µL of the diluted samples at a concentration of 10 ng/µL and 5 µL of the positive control, or nuclease-free water (negative control) to the corresponding wells.

HFE282 Master Mix	HFE63 Master Mix	HFE65 Master Mix
S1	S1	S1
S2	S2	S2
S3	S3	S3
PC	**PC**	**PC**
NTC	**NTC**	**NTC**

Figure 1. Example of the PCR template. S (samples), NTC (no template control), PC (positive control).

06 Place the tubes or plates in the real-time PCR thermal cycler and set up the amplification program as indicated in the following section.

07.2 | Real-time PCR program setup

- **Type of experiment**: Genotyping
- **Ramp speed**: Standard
- **Reaction volume**: 25 µL
- **ROX™ baseline reference**: included
- **Fluorophores of TaqMan® probes**:

PCR mix	Probe	Issuer	Genotyping	*Quencher*
HFE282	**HFE282-G-P**	VIC™	*Wild-type*	MGB
	HFE282-A-P	FAM™	c.845G>A	MGB
HFE63	**HFE63-C-P**	VIC™	*Wild-type*	MGB
	HFE63-G-P	FAM™	c.187C>G	MGB
HFE65	**HFE65-A-P**	VIC™	*Wild-type*	MGB
	HFE65-T-P	FAM™	c.193A>T	MGB

Table 2. Probe information

- **Optimal program**:

Fields	Stage 1 Pre-PCR reading	Stage 2 Enzymatic activation	Stage 3 PCR		Stage 4 Post-PCR reading
No. of cycles	1 cycle	1 cycle	50 cycles		1 cycle
			Denaturation	Oligonucleotide binding/extension	
Temperature	60°C	95°C	95°C	60°C	60°C
Time	1 minute*	10 minutes	15 seconds	1 minute*	1 minute*

Table 3. Optimal PCR program for the 7500 FAST and StepOne (ThermoFisher Scientific).

(*) Fluorescence detection

08 Analysis of results

It is recommended to follow the indications below for the results to be analyzed properly:

- Check that there is no amplification in the negative PCR control, neither in the FAM channel nor in the VIC channel.
- Check that there is an amplification signal in the positive control, both in the FAM channel and the VIC channel.
- In order to analyze the samples, specific software for the real-time PCR thermal cycler employed must be used.

The possible results obtained with the Imegen® HFE kit are shown below:

↘ Homozygous wild-type sample:

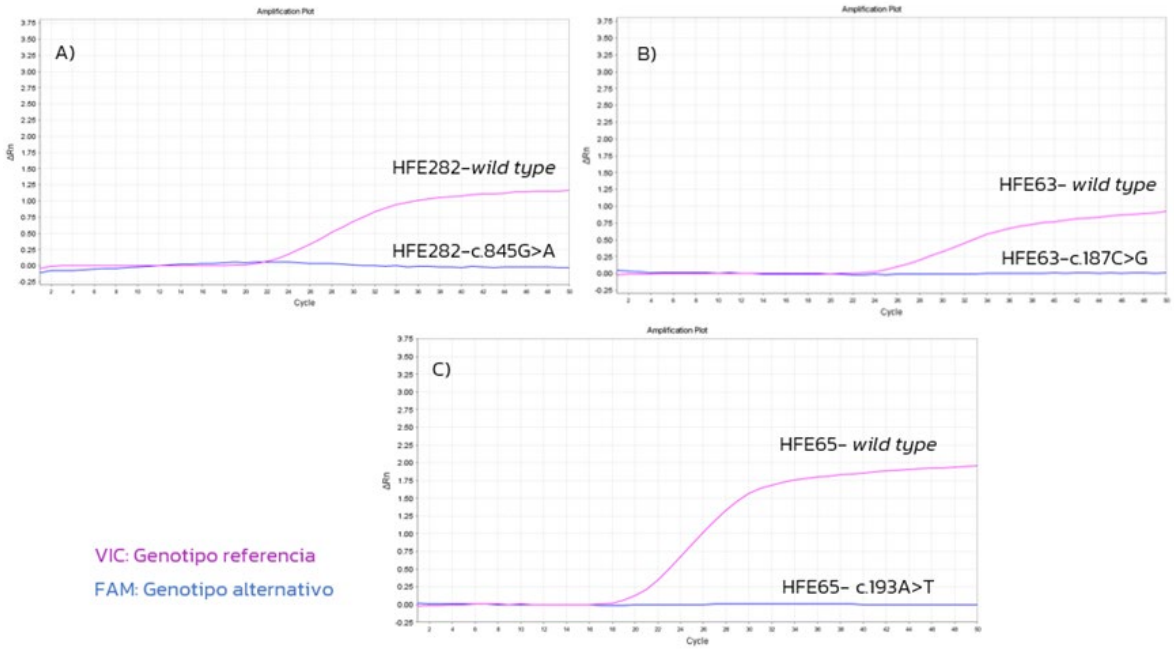

Figure 2. ***A)*** *Result obtained from a normal homozygous sample (G/G) for the HFE282 system.* ***B)*** *Result obtained from a normal homozygous sample (C/C) for the HFE63 system.* ***C)*** *Result obtained from a homozygous sample (A/A) for the HFE62 system. Amplification is only observed in the VIC channel.*

↘ Heterozygous sample:

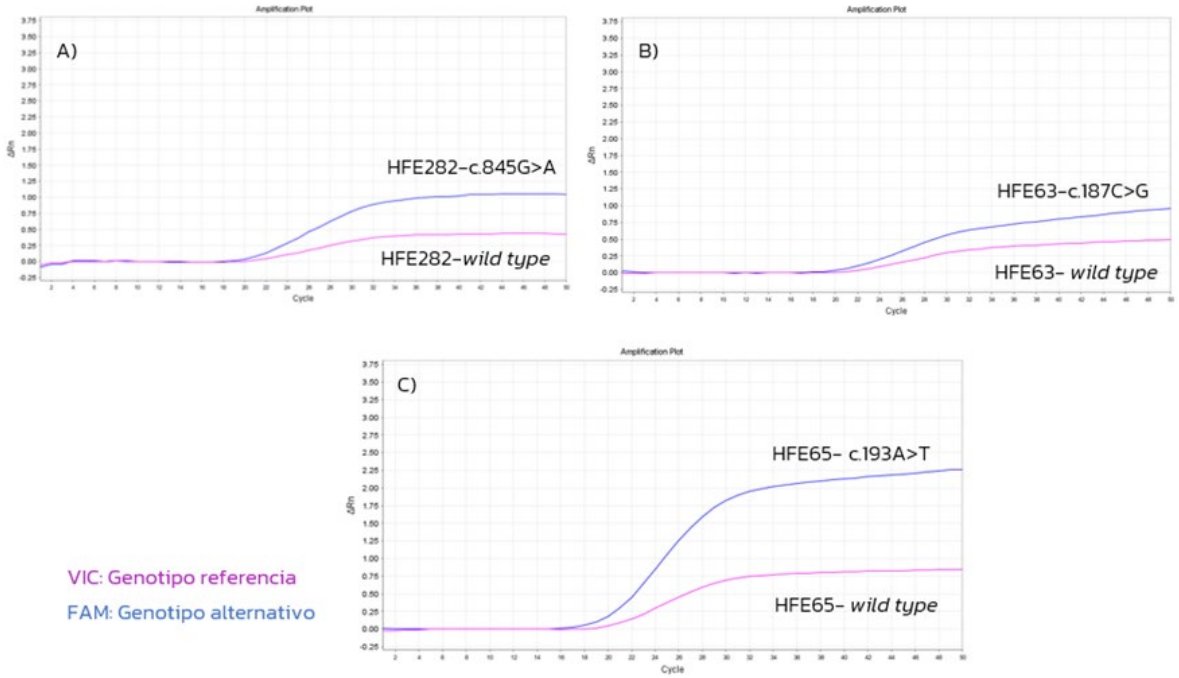

Figure 3. ***A)*** *Result obtained from a heterozygous sample (G/A) for the HFE282 system.* ***B)*** *Result obtained from a heterozygous sample (C/G) for the HFE63 system.* ***C)*** *Result obtained from a heterozygous sample (A/T) for the HFE65 system. Signal is observed in both FAM and VIC channels, the fluorescence intensity being higher in the FAM channel.*

↘ Homozygous mutant sample:

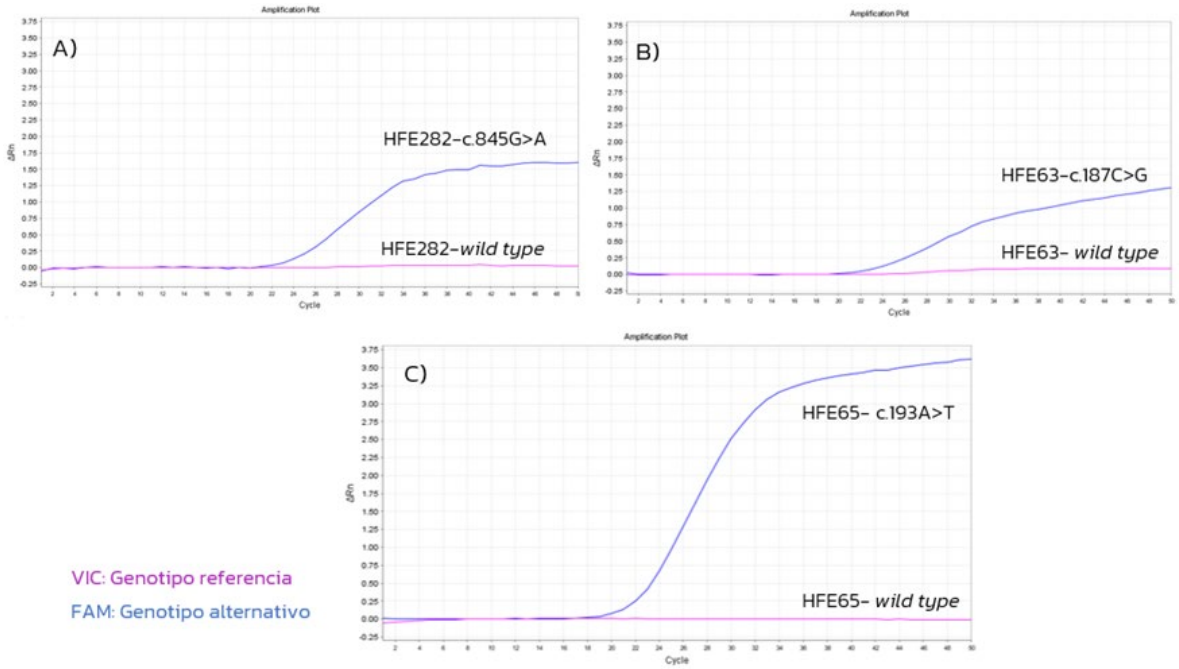

Figure 4. ***A)*** *Result obtained from a homozygous mutant sample (A/A) for the HFE282 system.* ***B)*** *Result obtained from a homozygous mutant sample (G/G) for the HFE63 system.* ***C)*** *Result obtained from a homozygous mutant sample (T/T) for the HFE65 system. Amplification is only observed in the FAM channel.*

↘ Allelic discrimination plots:

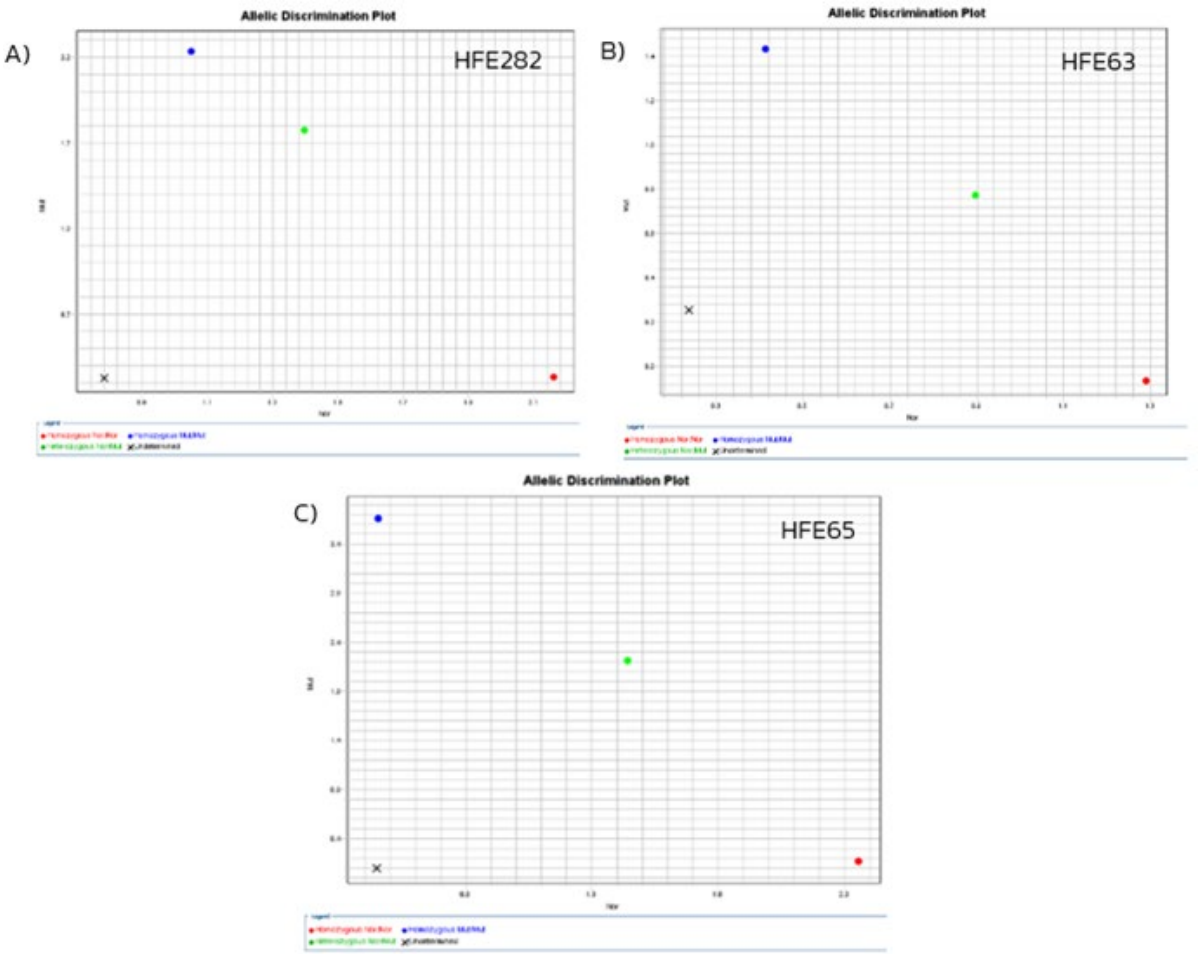

Figure 5. ***A)*** *Allelic discrimination plot with the HFE282 system.* ***B)*** *Allelic discrimination plot with the HFE63 system.* ***C)*** *Allelic discrimination plot with the HFE65 system. Homozygous normal samples are shown in red, heterozygous in green and homozygous mutants in blue in relation to all of the systems.*

09 Troubleshooting

The table below shows the results that could be obtained from the analysis of the different controls and a sample in an assay, as well as their interpretation.

Control	Result		Cause
	FAM	VIC	
Positive control	+	+	Expected result
	–	–	Failed PCR setup[1]
	+	–	
	–	+	
Sample	+	+	Expected result
	+	–	
	–	+	
	–	–	Failed sample amplification[2]
PCR negative control	–	–	Expected result
	+	+	Contamination of PCR with human DNA[3]
	+	–	
	–	+	

Table 4. Interpretation of possible results

(1) Failed PCR amplification: Check the amplification program and fluorescence capture settings. Failed amplification may be due to a technical problem in the PCR program settings.

(2) Failed sample amplification: Check that the quantification of the sample is as recommended. If so, the specified result may be due to the sample being highly degraded.

(3) PCR contamination with human DNA: PCR contamination may be due to mishandling of the sample, the use of contaminated reagents or contamination of environmental origin. Thoroughly clean the laboratory where the PCR was prepared, as well as the equipment and materials used. If necessary, use new aliquots of PCR reagents. Prepare the PCR reaction containing the positive control as the final step, in order to avoid cross-contamination. In this case, it is recommended to repeat the test.

10 Limitations

10.1 | Equipment

Imegen® HFE has been validated using the following PCR thermal cyclers:

- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Real-Time PCR System* (ThermoFisher Scientific)
- *StepOne Plus Real-Time PCR System* (ThermoFisher Scientific)

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

10.2 | Reagents

Imegen® HFE has been validated using the reagents included in the kit and those recommended in section 6 of this document (Equipment, reagents and materials not included in the kit).

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.

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

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