Instructions for use

Imegen® Alfa-1-AT

Ref. IMG-211

CE IVD

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

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Health in Code, S.L 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 Health in Code, S.L are subjected to rigorous quality control. Imegen® Alfa-1-AT 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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* **Imegen®** is a registered trademark in Spain of the Health in Code group

Instructions for Use (IFU) modifications		
Version 08	DEC 2022	Changing the storage and shipping temperature of the GENERAL MASTER MIX reagent (Section 5).
Version 07	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 06	SEP 2022	Change in manufacturer's identification: from Imegen to HEALTH IN CODE, S.L.
Version 05	FEB 2020	Type of experiment Quantitation-Standard Curve (page 11).

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01 General information

The SERPINA1 (NM_001002235) gene, located in chromosomal region 14q32.1, encodes the alpha-1-antitrypsin (AAT) protein, also known as a protease inhibitor (PI). The most important inhibitory action of AAT is against neutrophil elastase, a protection normally released to fight infections, but if it is not controlled by ATT it can degrade the elastin of the alveolar walls, as well as other protective proteins a variety of tissues.

Mutations in the SERPINA1 gene cause deficiency in AAT, an autosomal recessive disorder, the most common manifestation of which is mainly associated with the risk of emphysema, which is evident from the third decade. A less common manifestation of ATT deficiency is liver disease that occurs in children and adults and can cause cirrhosis and liver failure.

References

- *https://www.omim.org/entry/107400*
- *https://www.omim.org/entry/613490*

02 Intended use

Imegen® Alfa-1-AT kit uses a combination of oligonucleotides and fluorescent hydrolysis probes in a validated real-time PCR assay to detect the Glu342Lys (PI-Z; rs28929474; c.1096_G> A) and Glu264Val (PI -S; rs17580; c.863_A> T) of the SERPINA1 gene. In addition, it uses as a synthetic positive control that contains a v/v copy of the mutated allele for each mutation and a copy with the normal alleles, for qualitative analysis.

Imegen® Alfa-1-AT can be used for *in vitro* diagnosis and is aimed at professionals working in molecular biology.

03 Technical characteristics

This kit has been validated using samples previously genotyped by the Genetic Medicine department at Health in Code S.L and synthetic DNA samples with the target sequences (wildtype or mutant). The validation indicates the assay is highly sensitive and specific.

The material needed for this study is genomic DNA from peripheral blood. The total amount of DNA needed is 100 ng.

Health in Code, S.L. is certified under **UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems - Requirements for regulatory purposes** standard 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

- Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
- You must properly protect any skin condition, as well as cuts, abrasions, and other skin lesions.
- 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.
- In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.
- The materials safety data-sheets (MSDS) of all hazardous components contained in this kit are available on request to Health in Code, S.L.
- This product requires the handling of samples and materials of human origin. You should consider all human source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of blood borne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.
- Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.
- 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.
- 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 Health in Code, S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Health in Code, S.L, considering them equivalent to those provided in the Kit.

05 Content and storage conditions of the kit

This kit contains enough reagents for 48 determinations. The reagents included in the kit are as follows:

- **PI-S Master Mix**: contains oligonucleotides, fluorescent hydrolysis probes (FAM and VIC) and water for amplification and detection of normal (c.863A) and mutant (c.863T) alleles under analysis.
- **PI-Z Master Mix**: contains oligonucleotides, fluorescent hydrolysis probes (FAM and VIC) and water for amplification and detection of normal (c.1096G) and mutant (c.1096A) alleles under analysis.
- **General Master Mix**: PCR Master Mix with nucleotides, $MgCl_2$, DNA polymerase and enzyme buffer to carry out the real-time PCR.
- **Positive Control**: heterozygous sample for both target mutations.

Reagents	Color indicator	Volume	Conservation
PI-S Master Mix	Red disk	2 x 180 µl	-20°C
PI-Z Master Mix	Blue disk	2 x 180 µl	-20°C
General Master Mix	White disk	1320 µl	-20°C*
Positive Control	Red lid	1 x 200 µl	-20°C

Table 1. Components of the Imegen® Alfa-1-AT kit

(*) **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 material not included in the kit

Equipment:

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

Reagents:

- Nuclease-free wáter

↘ For the **Lightcycler 480 & Capillar Lightcycler** (Roche):

  - LightCycler FastStart DNA Master HybProbe
  - $MgCl_2$ (25 mM)

Materials:

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

07 Assay protocol

07.1 | Preparation of the PCR reagents

To estimate the number of reagents needed, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend performing the calculations, considering one more reaction, or increasing the volume of each reagent by 10%.

To carry out the qualitative analysis, two amplification reactions will be necessary, one for each mutation. It is recommended to prepare a sample amplification reaction and include a negative PCR control, to rule out contamination of the reagents and the positive control.

Then, regardless of the real-time PCR thermal cycler you have, place the tubes, plates or capillaries in the real-time PCR thermal cycler. To configure the amplification program, it is necessary to consider the fluorophore of the probes used (see Table 2).

Probe	Fluorophore	Genotype	Quencher
PIS-A	VIC™	Wildtype	MGB
PIS-T	FAM™	Mutant	
PIZ-G	VIC™	Wildtype	
PIZ-A	FAM™	Mutant	

Table 2. Hydrolysis probe information

The following is the recommended protocol for the preparation of the amplification reactions:

01 Allow all reagents and DNA samples to thaw.

02 Vortex the reagents and spin down. Keep cold until needed.

7500 FAST, StepOne or StepOne Plus (ThermoFisher Scientific)

03 In 1.5 mL tubes, one for the analysis of each mutation, add the volumes according to Table 3:

Reagents	Volume per reaction
PI-S or PI-Z Master Mix	7.5 µL
General Master Mix	12.5 µL

Table 3. Volume of reagents needed per reaction with 7500 FAST or StepOne equipments.

04 Vortex and spin the PCR mix and dispense 20 µL into the corresponding wells of the fungible optical material or capillaries used.

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 of the optical or capillary fungible material used.

Lightcycler 480 and capillary Lightcycler (Roche)

03 In 1.5 mL tubes, one for the analysis of each mutation, add the necessary amounts of reagents from Table 4:

Reactivos	Volumen por reacción
PI-S o PI-Z Master Mix	4 µL
LightCycler FastStart DNA Master HybProbe	1 µL
$MgCl_2$ (25 mM)	1 µL
Nuclease-free water	9 µL

Table 4. Volume of reagents needed per reaction with LightCycler 480 and Capillar.

04 Vortex and spin the PCR mix and dispense 15 µL into the corresponding wells of the fungible optical material or capillaries used.

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 of the optical or capillary fungible material used.

07.2 | Setup of the PCR programme

7500 FAST, StepOne or StepOne Plus (Thermo Scientific)

- **Type of experiment**: Quantitation —Standard curve
- **Ramp speed**: Standard
- **Reaction volume**: 25 µL
- **Reference ROX™ 7500 FAST and StepOne**
- **Optimal program**:

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

Table 5. Optimal programme for 7500 FAST and StepOne (Thermo Scientific)

(*) Fluorescence detection

Lightcycler 480 Real-time PCR cycler (Roche)

- **Experiment**: Dual Color Hydrolysis Probe / UPL Probe
- **Reaction volume**: 25 µL
- **Analyses**: Abs Quant / Fit Points
- **Optimal program**:

Fields	Phase 1 Enzymatic activation	Phase 2 PCR			Phase 3
No. of cycles	1 initial cycle	50 cycles			1 final cycle
		Denaturation	Annealing	Extension	
Temperature	95°C	95°C	60°C	72°C	40°C
Time	10 minutes	5 segundos	10 segundos	15 segundos*	20 segundos

Table 6. Optimal programme for LightCycler 480 real-time PCR cycler

(*) Fluorescence detection

Capillary Lightcycler Real-time PCR cycler (Roche)

- **Samples>Selected Channels**: Select 530 and 560
- **Optimal program**:

Programs						
Program Name				Cycles		Analysis Mode
Preheating				1		None
Quantification				50		Quantification
Cold				1		None
Preheating Temperature Targets						
Target (°C)	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
95	00:10:00	20	0	0	0	None
Quantification Temperature Targets						
Target (°C)	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
95	00:00:02	20	0	0	0	None
60	00:00:12	20	0	0	0	Single
72	00:00:08	20	0	0	0	None
Cold Temperature Targets						
Target (°C)	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
40	00:00:30	20	0	0	0	None

Table 7. Optimal PCR program for Capillary LightCycler.

08 Analysis of results

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 in positive control (Alfa-1-AT control) wells there is positive signal in both, FAM and VIC channels for both master mixes (PI-S and PI-Z PCR master mixes).
- Specific software has to be used to analyze the samples in this type of analysis. If manual assay is performed considering fluorescence increments, it must be considered the following points:

The possible results obtained with Imegen® Alfa-1-AT using different real-time PCR thermal cyclers are as follows:

RESULT INTERPRETATION WITH **7500 FAST** AND **STEPONE**

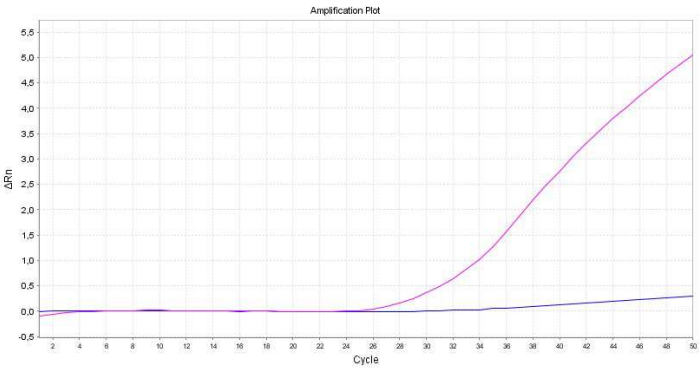

Figure 1. Result obtained from a normal homozygous sample (A / A) for the PI-S mutation. A clear amplification is observed in the VIC channel and a residual amplification signal in the FAM channel

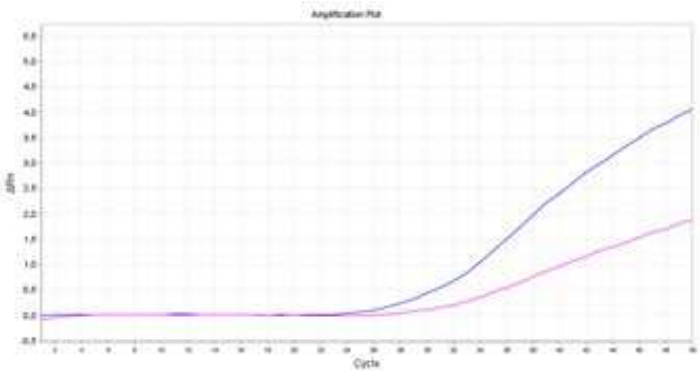

Figure 2. Result obtained from a heterozygous (A/T) sample for the PI-S mutation. Signal is observed in both channels, FAM and VIC, the fluorescence intensity being higher in the FAM channel

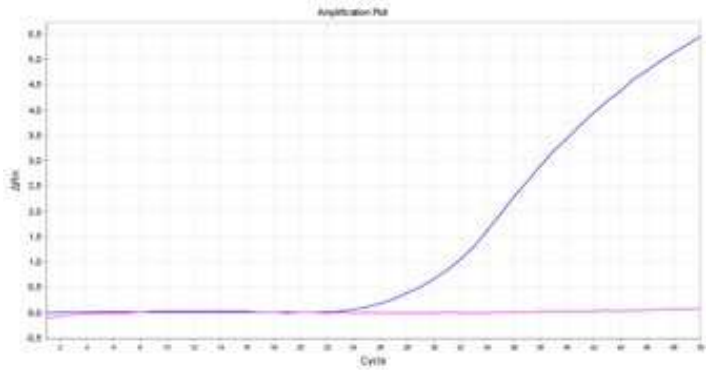

Figure 3. Result obtained from a homozygous mutant sample (T / T) for the PI-S mutation. Only amplification is observed in the FAM channel

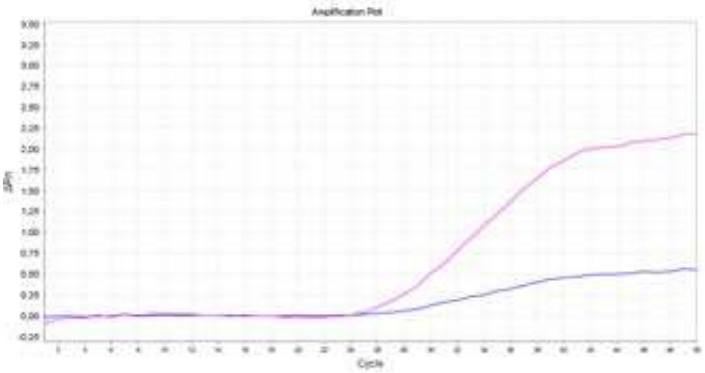

Figure 4. Result obtained from a normal homozygous (G / G) sample for the PI-Z mutation. A clear amplification is observed in the VIC channel and a residual amplification signal in the FAM channel

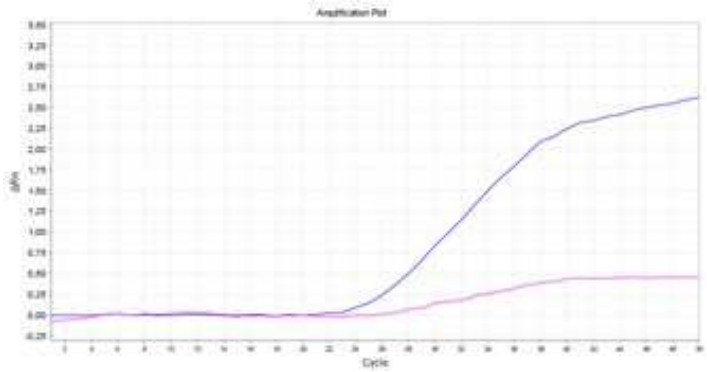

Figure 5. Result obtained from a heterozygous (G / A) sample for the PI-Z mutation. Signal is observed in both channels, FAM and VIC, the fluorescence intensity being higher in the FAM channel

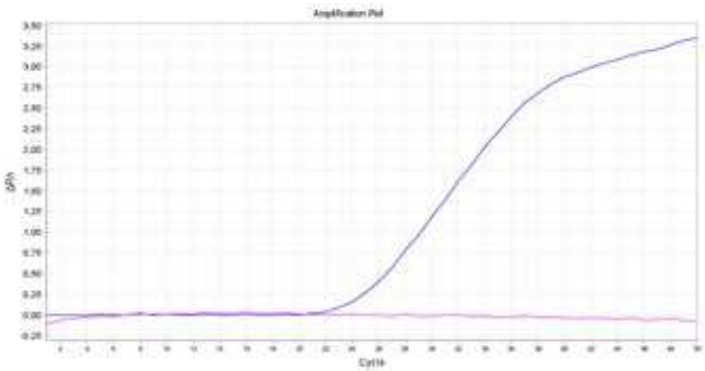

Figure 6. Result obtained from a homozygous mutant sample (A / A) for the PI-Z mutation. Only amplification is observed in the FAM channel

RESULT INTERPRETATION WITH **LIGHTCYCLER**

PI-S system, FAM channel (530 nm)

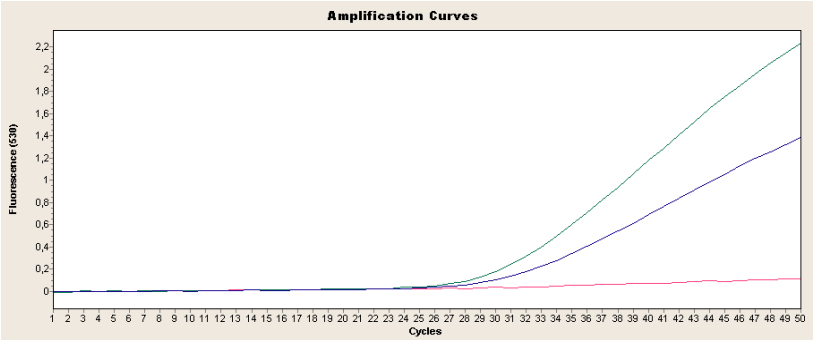

Figure 7. Results obtained. In the normal homozygous (A / A) (green) and in the heterozygous (A / T) (blue) a clear amplification is observed in the FAM channel. In the homozygous mutant (T / T) (pink) there is no amplification in the FAM channel

PI-S system, VIC channel (560 nm)

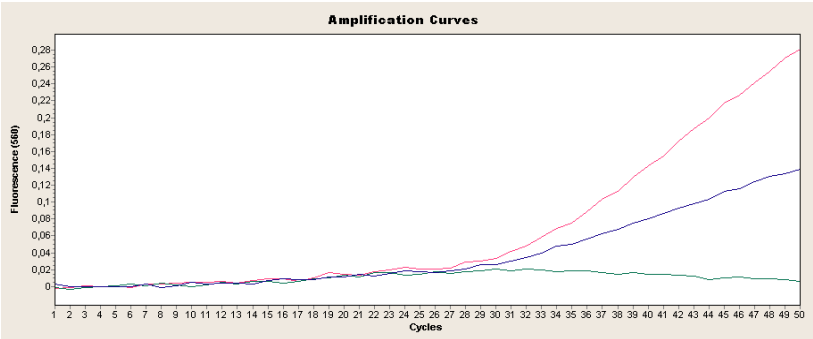

Figure 8. Results obtained. In the normal homozygous (A / A) (green) no amplification is observed in the VIC channel. In the heterozygous (A / T) (blue) and in the homozygous mutant (T / T) (pink) a clear amplification is observed in the VIC channel

PI-Z system, FAM channel (530 nm)

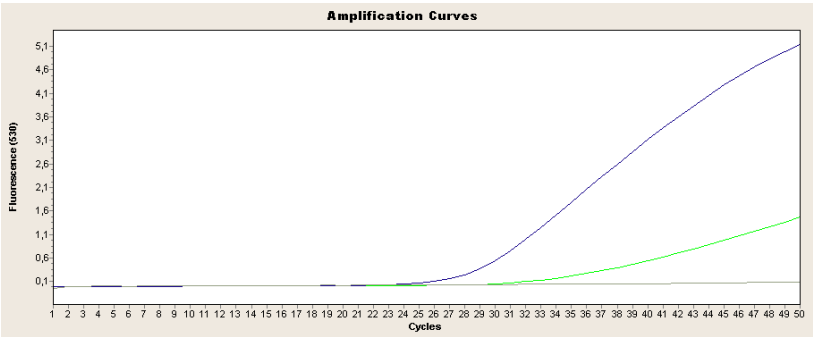

Figure 9. Result obtained from a normal homozygous sample (G / G). A residual amplification signal is observed on the FAM channel (green)

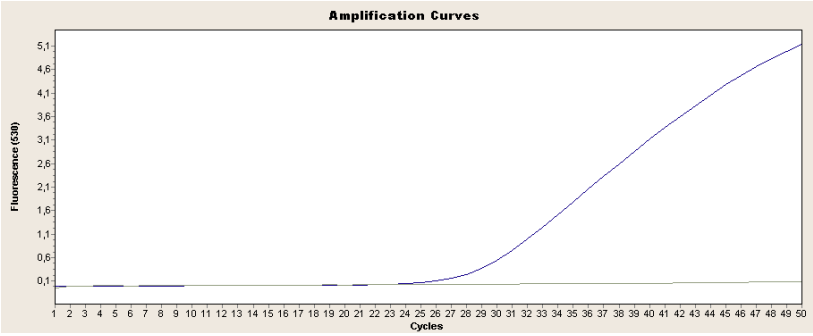

Figure 10. Result obtained from a heterozygous sample (G / A). Clear amplification in the FAM channel

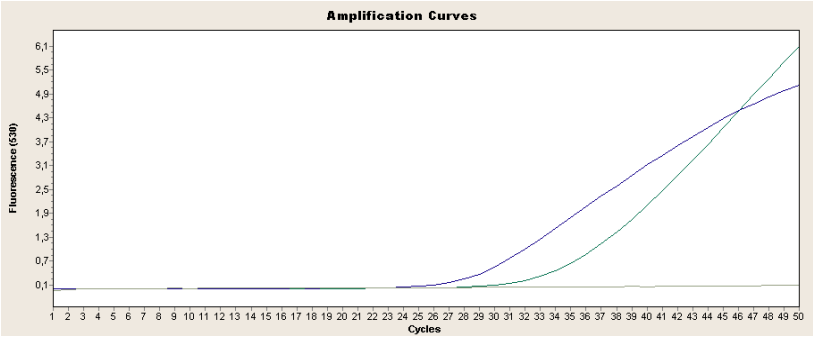

Figure 11. Result obtained from a homozygous mutant sample (A / A). Clear amplification in the FAM channel

PI-Z system, VIC channel (560 nm)

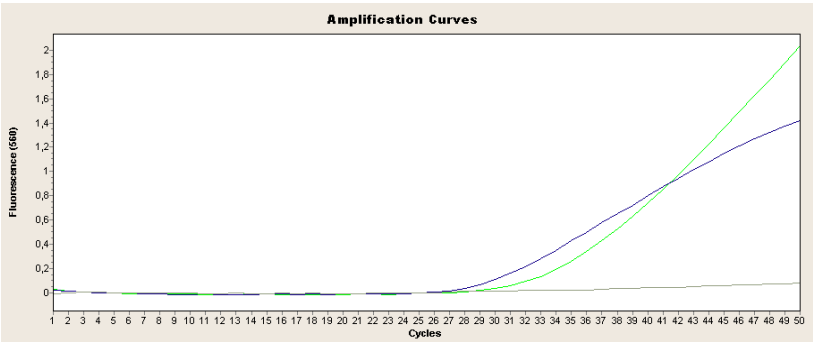

Figure 12. Result obtained from a normal homozygous sample (G / G). Clear amplification in the VIC channel (green)

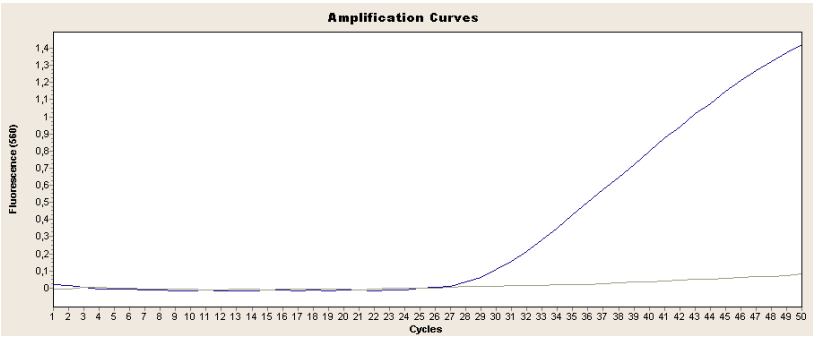

Figure 13. Result obtained from a heterozygous sample (G / A). Clear amplification in the VIC channel

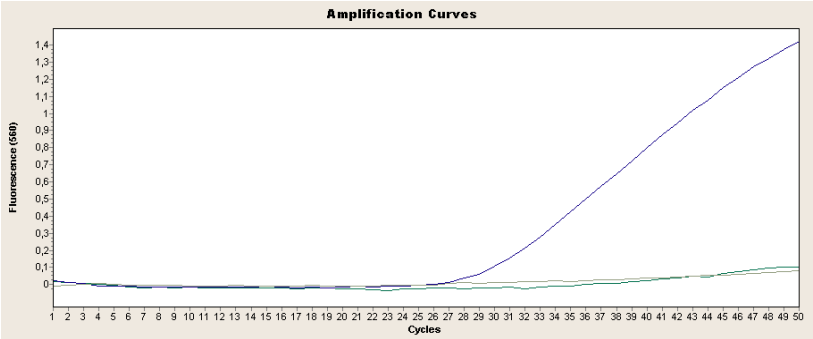

Figure 14. Result obtained from a homozygous mutant sample (A / A). Absence of amplification in the VIC channel (green)

09 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 in both PCR systems:

Control	Result		Result / Interpretation
	FAM	VIC	
Positive control	+	+	Expected result
	–	–	PCR amplification failure [1]
Sample	+	+	Expected result
	+	–	
	–	+	
	–	–	Sample amplification failure [2]
Negative control	–	–	Expected result
	+	+	PCR contamination with human DNA [3]
	+	–	
	–	+	

Table 7. Results interpretation

(1) PCR amplification failure: check the amplification program and fluorescence capture settings. A failure in the amplification may be due to a technical problem in the configuration of the PCR program.

(2) Sample amplification failure: check that the quantification of the sample is recommended, if so, the specified result may be due to the highly degraded sample.

(3) PCR contamination with human DNA: the contamination of the PCR may be due to the wrong handling of the sample, the use of contaminated reagents or contamination of environmental origin. Thoroughly clean the laboratory where the PCR has been prepared, as well as the equipment and equipment used. If necessary, use new aliquots of the PCR reagents. Finally prepare the PCR reaction that contains the positive control, to avoid cross contamination. In this case it is recommended to repeat the test.

10 Limitations

10.1 | Equipment

Imegen® Alfa-1-AT 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)
- *LightCycler Capillar* (Roche)
- *LightCycler 480* (Roche)

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

10.2 | Reagents

Imegen® Alfa-1-AT 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).

10.3 | Product stability

The optimal operation of this product is confirmed provided that the recommended storage conditions specified within the optimal date of the product associated with each production lot are applied.

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

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