Instructions for use

Imegen® Alfa-1-AT

Ref. IMG-211

CE IVD

MAnufactured by:
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Code: HIC-PT-KIT 03-F-03 V.02

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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® Alfa-1-AT 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 (IFU)		
Version 09	DEC 2023	Review and update of section "3. Technical characteristics".
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 S.L. to Health in Code S.L.
Version 05	NOV 2018	Type of experiment, Quantitation-Standard Curve in section 7.2, Real-time PCR program setup for the 7500 Fast and StepOne

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01 General information

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

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

References

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

02 Intended use

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

Imegen® Alfa-1-AT is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

This kit has been validated using samples previously analyzed by the medical genetics service of Health in Code, S.L., and synthetic DNA with a single copy of the target sequences (normal or mutant) of the regions under analysis of the *SERPINA1* gene, and specifically detects the expected genotypes.

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

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.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, 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 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.

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:

- *PI-S Master Mix:* contains oligonucleotides, fluorescent hydrolysis probes (FAM and VIC) and water for amplification and detection of the normal and/or mutant alleles under analysis.
- *PI-Z Master Mix:* contains oligonucleotides, fluorescent hydrolysis probes (FAM and VIC) and water for amplification and detection of the normal and/or mutant alleles under analysis.
- *General Master Mix*: PCR Master Mix with nucleotides, $MgCl_2$, enzyme and buffer to perform real-time PCR.
- *Positive Control:* positive control for the simultaneous amplification of the normal and mutant alleles under analysis (simulating a heterozygous sample for both mutations).

Reagents	Color	Quantity	Storage
PI-S Master Mix	Red pad	2 x 180 µL	-20°C
PI-Z Master Mix	Blue pad	2 x 180 µL	-20°C
General Master Mix	White pad	1320 µL	-20°C
Positive control	Red cap	1 x 200 µL	-20°C

Table 2. Imegen® Alfa-1-AT 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

Reagents:

- Nuclease-free wáter

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

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

Materials:

- Pipette tips with filter (10 µL, 20 µL, 200 µL)
- 1.5 mL sterile tubes.
- 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
- Latex gloves

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.

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

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

01 Thaw all kit reagents and DNA from the samples. Vortex each of the reagents and keep cold.

Then, regardless of which real-time PCR thermal cycler you are using, place the tubes, plates or capillaries in the real-time PCR thermal cycler. It is necessary to consider the fluorophores of the probes used (see table 4) to configure the amplification program.

Probe	Recipient	Genotyping	Emitter or Quencher
PIS-A-P	VIC®	Normal	MGB
PIS-T-P	FAM™	Mutant	MGB
PIZ-G-P	VIC®	Normal	MGB
PIZ-A-P	FAM™	Mutant	MGB

Table 4. Probe information

Then, depending on the real-time PCR equipment you are using, follow points 2-4:

PCR in 7500 FAST, StepOne or StepOne Plus (Thermo Fisher Scientific)

02 Add the required quantities of reagents in table 2 to 1.5 mL tubes, one for each mutation analysis:

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

Table 2. Quantity of reagents required per reaction to use 7500 FAST or StepOne.

03 Vortex and spin the PCR mix, then dispense 20 µL into the corresponding wells of the optical consumables or capillaries used.

04 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 consumables used.

LightCycler 480 or capillary LightCycler (Roche)

02 Add the required quantities of reagents in table 3 to 1.5 mL tubes, one for each mutation analysis:

Reagents	Volume per reaction
PI-S or PI-Z Master Mix	4 µL
LightCycler FastStart DNA Master HybProbe	1 µL
$MgCl_2$ (25 mM)	1 µL
Water	9 µL

Table 3. Required quantity of reagents per reaction using a LightCycler 480 or capillary LightCycler.

03 Vortex and spin the PCR mix, then dispense 15 µL into the corresponding wells of the optical consumables or capillaries used.

04 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 consumables used.

07.2 | Real-time PCR optimized program setup for 7500 Fast and StepOne

- **Type of experiment**: Quantitation – Standard curve
- **Ramp speed**: Standard
- **Reaction volume**: 25 µL
- **ROX™ baseline reference for 7500 FAST and StepOne**: included
- **Optimal program**:

Fields	Stage 1 Enzymatic activation	Stage 2 PCR	
No. of cycles	1 initial cycle	50 cycles	
		Denaturation	Oligonucleotide 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 and StepOne (Thermo Fisher Scientific).

(*) Fluorescence detection

07.3 | Real-time PCR optimized program setup for capillary LightCycler (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 6. Optimal PCR program for capillary LightCycler (Roche).

07.4 | Real-time PCR optimized program setup for LightCycler 480 (Roche)

Fields	Stage 1 Enzymatic activation	Stage 2 PCR			Stage 3
No. of cycles	1 initial cycle	50 cycles			1 final cycle
		Denaturation	Oligonucleotide 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 7. Optimal PCR program for the LightCycler 480 thermal cycler.

(*) 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 both systems.
- 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® Alfa-1-AT kit using different thermal cyclers are shown below.

08.1 | Possible results obtained with 7500 Fast and StepOne

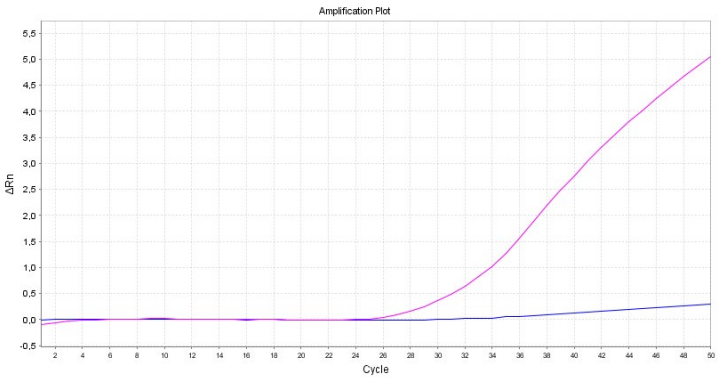

Figure 1. Result obtained from a normal homozygous sample (A/A) for the PI-S mutation. Clear amplification is observed in the VIC channel and a residual amplification signal in the FAM channel.

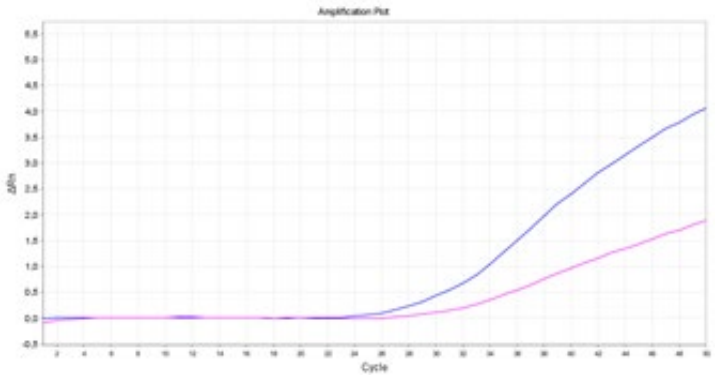

Figure 2. Result obtained from a heterozygous sample (A/T) for the PI-S mutation. Signal is observed in both FAM and VIC channels, the fluorescence intensity being higher in the FAM channel.

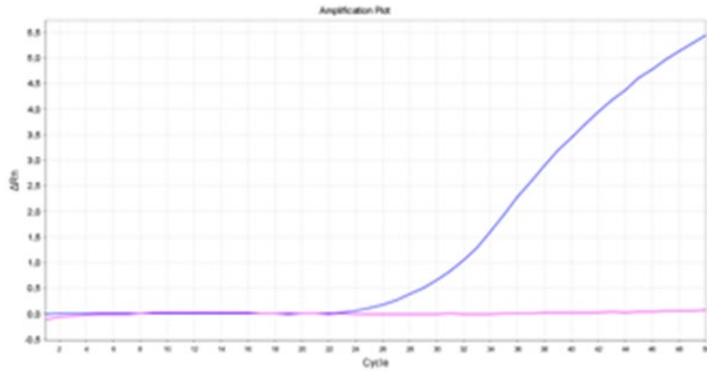

Figure 3. Result obtained from a homozygous mutant sample (T/T) for the PI-S mutation. Amplification is only observed in the FAM channel.

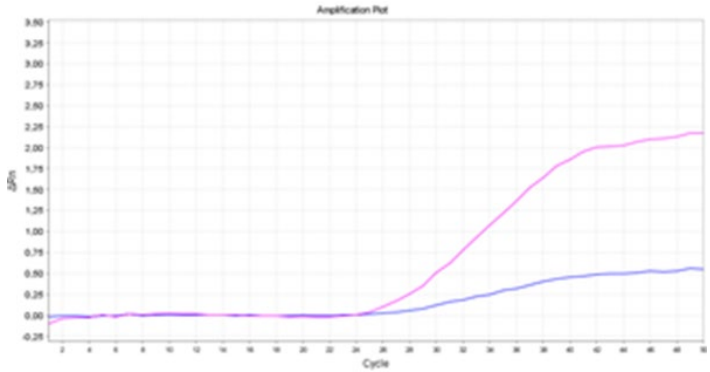

Figure 4. Result obtained from a normal homozygous sample (G/G) for the PI-Z mutation. Clear amplification is observed in the VIC channel and a residual amplification signal in the FAM channel.

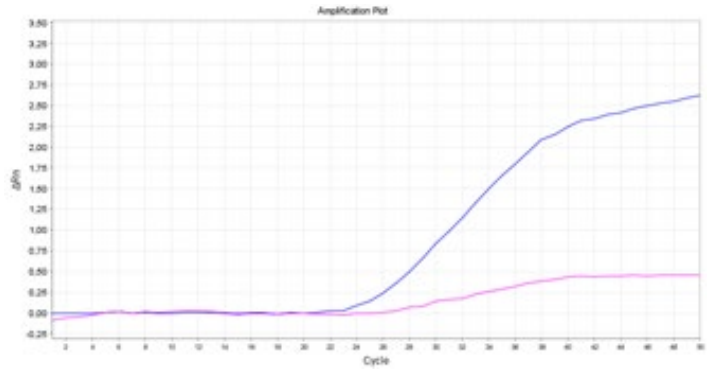

Figure 5. Result obtained from a heterozygous sample (G/A) for the PI-Z mutation. Signal is observed in both FAM and VIC channels, the fluorescence intensity being higher in the FAM channel.

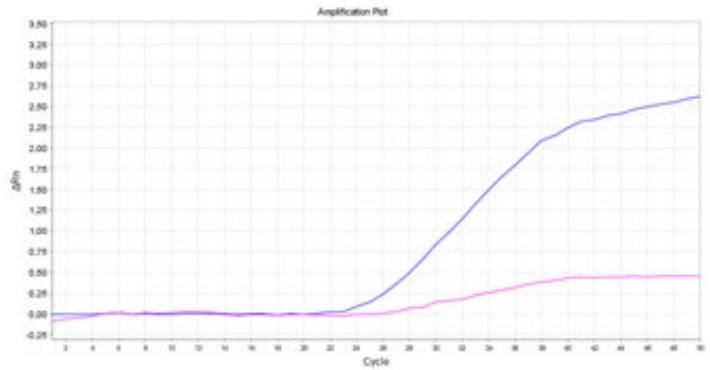

Figure 6. Result obtained from a homozygous mutant sample (A/A) for the PI-Z mutation. Amplification is only observed in the FAM channel.

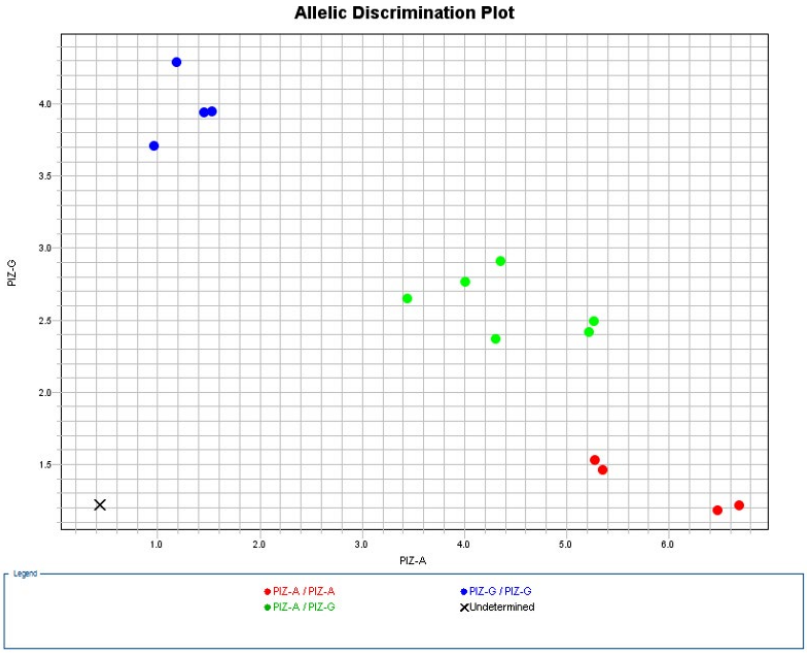

Figure 7. Example of the allelic discrimination graph (PI-Z system).

08.2 | Possible results obtained with LightCycler

PI-S system, FAM channel (530 nm)

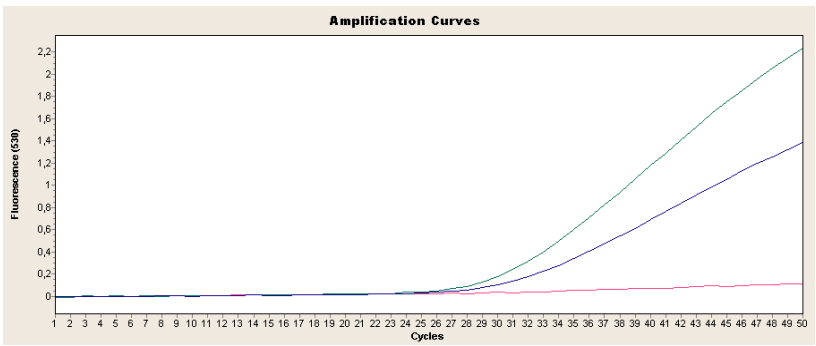

Figure 8. Results obtained Clear amplification in the FAM channel is observed in the normal homozygote (A/A) (green) and the heterozygote (A/T) (blue). There is no amplification in the FAM channel in the homozygous mutant (T/T) (pink).

PI-S system, VIC channel (560 nm)

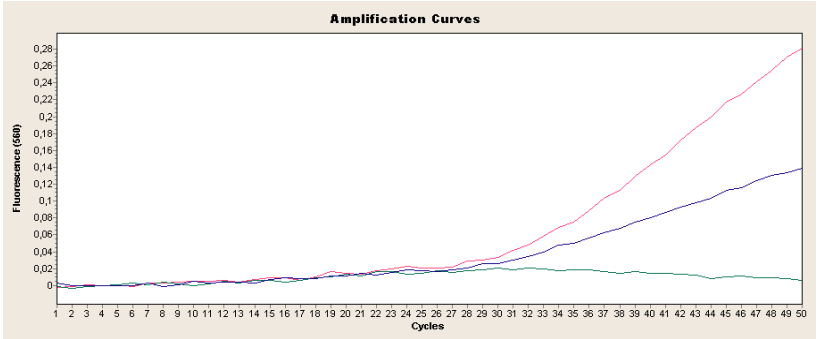

Figure 9. Results obtained No amplification is observed in the VIC channel in the normal homozygote (A/A) (green). Clear amplification in the VIC channel is observed in the heterozygote (A/T) (blue) and the mutant homozygote (T/T) (pink).

PI-Z system, FAM channel (530 nm)

The signal from the positive control included in the kit (blue) is included in all the results figures.

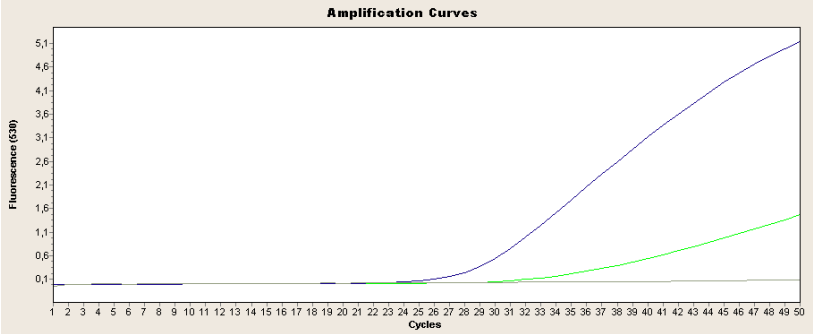

Figure 10. Result obtained from a normal homozygous sample (G/G). A residual amplification signal is observed in the FAM channel (green).

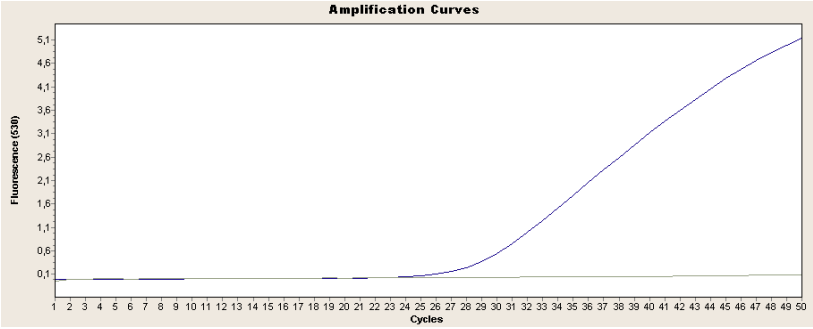

Figure 11. Result obtained from a heterozygous sample (G/A). Clear amplification in the FAM channel.

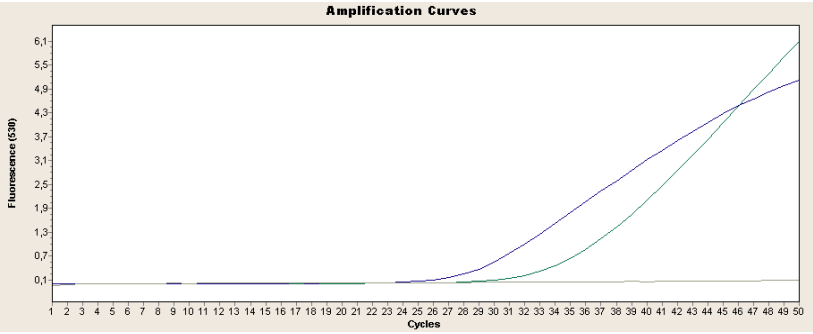

Figure 12. Result obtained from a homozygous mutant sample (A/A). Clear amplification in the FAM channel.

PI-Z system, VIC channel (560 nm)

The signal from the positive control included in the kit (blue) is included in all the results figures.

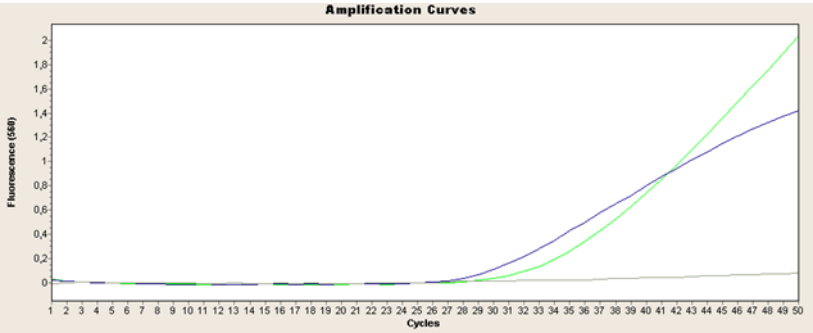

Figure 13. Result obtained from a normal homozygous sample (G/G). Clear amplification in the VIC channel (green).

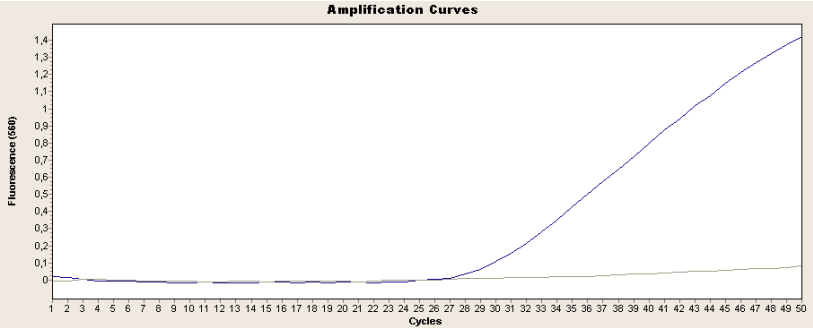

Figure 14. Result obtained from a heterozygous sample (G/A). Clear amplification in the VIC channel.

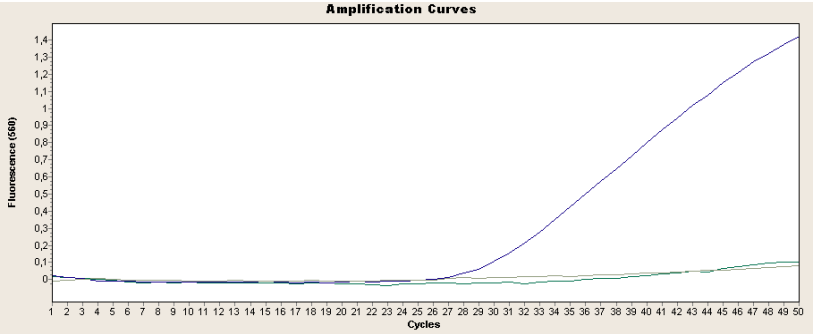

Figure 15. Result obtained from a homozygous mutant sample (A/A). No amplification in the VIC channel (green).

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 in both PCR systems:

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

Table 5. Interpretation of possible results with the Imegen® Alfa-1-AT kit

(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® Alfa-1-AT has been validated using the following PCR thermal cyclers:

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

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® Alfa-1-AT has been validated using the reagents included in the kit and those recommended in section 6 of this manual (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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