

## Elucigene<sup>®</sup> AAT

**Catalogue Code: AA002B2**

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Elucigene kits are developed and manufactured by Gen-Probe Life Sciences Ltd. within quality systems accredited to ISO9001:2008 and ISO13485:2003.

Manufactured by:  
Gen-Probe Life Sciences Ltd.  
Oaks Business Park  
Crewe Road  
Wythenshawe  
Manchester  
M23 9HZ

For Sales, Customer Service and Technical Support:-

T: +49 (0) 6122 7076451

F: +49 (0) 6122 7076155

E: [customerservice@gen-probe.eu](mailto:customerservice@gen-probe.eu)

E: [technicalsupport@gen-probe.eu](mailto:technicalsupport@gen-probe.eu)



# Elucigene<sup>®</sup> AAT

Catalogue Code: AA002B2 – 50 tests

## Intended Use

For the simultaneous *in vitro* qualitative detection of PI\*S and PI\*Z alleles of the human Alpha-1-Antitrypsin (AAT) gene in DNA extracted from whole blood (EDTA preserved) or dried bloodspot samples.

## Summary and Explanation

Alpha-1-Antitrypsin (AAT) deficiency is an autosomal recessive disorder virtually confined to Caucasians of European descent and is the most common genetic cause of liver disease in children <sup>(1)</sup> and pulmonary emphysema in adults <sup>(2)</sup>.

Alpha-1-Antitrypsin (AAT) is a circulating serine protease inhibitor (PI) encoded by the PI locus (HUGO symbol; SERPINA1), located on chromosome 14q, which inhibits the activity of proteolytic enzymes, its main target being neutrophil elastase particularly in the lower respiratory tract. A deficiency of AAT results in an elevated neutrophil elastase activity in the lung which causes alveolar wall damage. The resultant chronic obstructive pulmonary disease (COPD) is the most prevalent clinical manifestation of AAT deficiency, although symptoms of liver abnormalities can also be expressed in infancy <sup>(3,4)</sup>. In AAT deficient non-smokers, the first symptoms of lung disease occur at a mean age of 45 years; however, in smokers that is lowered to 35 years of age. AAT deficient smokers also show a considerably increased rate of lung destruction and have a poorer survival rate than non-smokers <sup>(5)</sup>.

Current testing for AAT deficiency generally involves a routine automated biochemical screening test to identify patients with low serum levels of the AAT glycoprotein. This is followed by isoelectric focusing (IEF), a technique for separating and identifying protein variants. Many variants of the AAT gene have been identified but relatively few cause severe AAT deficiency. There are three major variants – M, Z and S. The M variant is normal and is classified into subtypes. The Z and S variants are caused by point mutations in the AAT gene and are associated with a reduction in AAT plasma levels, in addition the Z protein does not function normally.

In Caucasian populations, AAT deficiency affects approximately 1 in 2500 people, it is estimated that there are 70,000 to 100,000 individuals affected in the United States, with comparable numbers in Europe <sup>(6)</sup>.

The two variants, PI\*Z and PI\*S, in particular are known to account for the majority of AAT deficiency with carrier frequencies in Caucasians of 0.3-4% and 0.6-11% respectively <sup>(7)</sup>. Elucigene AAT provides laboratories with a simple, robust test as a means of routinely detecting PI\*Z and PI\*S. The methodology is rapid and straightforward producing results which are easily interpreted. The test provides genotype information which clearly identifies an individual as heterozygous (i.e. has one copy) or homozygous (has two copies) for the PI\*Z and PI\*S alleles.

## Principles of the Procedure

The method employed by the Elucigene AAT test is based on Amplification Refractory Mutation System (ARMS), an allele specific PCR amplification technology which can detect point mutations or small deletions in deoxyribonucleic acid (DNA)<sup>(8)</sup>. The test comprises two complementary reaction mixes. The first reaction mix specifically amplifies all AAT alleles which are unaffected by the S and Z mutations, i.e. wildtype. By contrast, the second reaction mix contains primers which specifically amplify only the S and Z mutant alleles. Each reaction mix also includes primers that amplify non-AAT DNA sequences as an assay internal amplification control to indicate a successful amplification. Amplified products (amplicons) of the two reactions are separated by electrophoresis on an agarose gel, presence or absence of bands on the gel indicate the status of PI\*S and PI\*Z alleles

## Warnings and Precautions

1. For *In Vitro* Diagnostic Use.
2. The DNA Control supplied with this kit is of human origin and has been independently tested using a PCR based assay and found to be negative for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus 1 (HIV1).
3. Care should be taken when handling material of human origin. All samples should be considered potentially infectious. No test method can offer complete assurance that HBV, HCV, HIV 1 or other infectious agents are absent. Handling of samples and test components, their use, storage and disposal should be in accordance with the procedures defined by the appropriate national biohazard safety guideline or regulation.
4. Store all components below -20°C. Discard 3 months after opening unless sub-aliquotted.
5. In line with current good laboratory practice, laboratories should process their own internal QC samples of known genotype in each assay, so that the validity of the procedure can be assessed.
6. Do not substitute or mix reagents from different kit lots or from other manufacturers.

## Symbols used on labels

The symbols used on all labels and packaging conform to the harmonised standard EN980



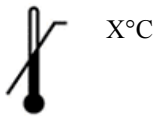
Manufacturer



Number of tests



See Instructions for Use



Store below temperature shown



Use before date shown



Catalogue code



Lot or batch number

## Materials Provided

Storage of the reagents should be in an area free from contaminating DNA or PCR product.

Do not use reagents beyond the expiry date stated on the package label. All reagents are supplied ready for use. Store unopened and opened reagents at -20°C. Opened reagents can be stored for up to 3 months.

Sufficient materials for 50 tests are provided:

1. 2 vials x 450µL of Primer Mix A (TA) containing primers specific for amplification of alleles unaffected by the S and Z mutations, control primers and deoxynucleotide triphosphates in buffer. (AA002TA)
2. 2 vials x 450µL of Primer Mix B (TB) containing primers specific for amplification of alleles affected by the S and Z mutations, control primers and deoxynucleotide triphosphates in buffer. (AA002TB)
3. 1 vial x 600µL of Loading Dye (LD). (CR000TR)
4. 1 vial x 200µL of Dilution Buffer (DB). (CR000TV)
5. 1 vial x 50µL of DNA Control (DC) is heterozygous for the S mutation detected by Elucigene AAT. (CR002TX)

## Materials required but not provided

Laboratory consumables - gloves; screw-capped microfuge tubes; pipette tips; thin-walled 0.2mL or 0.5mL PCR vials (use of two differently coloured vials will aid primer mix identification).

DNA Preparation - Good quality sterile deionised water; sodium chloride (NaCl); ethylenediaminetetra-acetic acid (EDTA) disodium salt; sodium hydroxide pellets (NaOH); 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base) crystallised; hydrochloric acid 36% sp.gr.1.18 (HCl); ammonium chloride (NH<sub>4</sub>Cl).

PCR Amplification - Sigma light white mineral oil\*; good quality sterile distilled water; AmpliTaq Gold (Applied Biosystems).

Electrophoresis - Gel electrophoresis materials including NuSieve® 3:1 Agarose (Lonza); 50 Base-Pair Ladder (GE Healthcare); Ethidium bromide.

\* For amplification carried out in 0.5mL PCR vials or thermal cyclers without heated lids

## Equipment Required

Laboratory equipment - precision pipettes (2 sets: 1 for pre-amplification and 1 for post-amplification handling:- preferably positive displacement pipettes); glassware; protective clothing; vortex mixer; microfuge; balance; tube racks.

DNA Preparation - Heating block (heating to 100°C).

Amplification - Thermal cycler to accommodate 0.5mL or 0.2mL vials (with a temperature accuracy of +/-1°C between 33°C and 100°C and static temperature uniformity of +/-1°C), heated lid optional.

Electrophoresis - Horizontal submarine gel tank; power pack; microwave; waterbath to cool agarose; UV transilluminator; photographic system.

### **Sample Collection and Storage**

Whole blood (EDTA) or bloodspots samples should be used.

Sample collection devices have on occasion been reported to be detrimental to the integrity of certain analytes and could interfere with some method technologies<sup>(9)</sup>. It is recommended that each user ensure that the chosen device is used according to the manufacturer's instructions and both sample collection devices and alternative DNA preparation methods are compatible with this test.

Blood samples should be stored at -20°C prior to preparation of DNA. Avoid repeated freezing and thawing.

### ***Preparation of DNA from Whole Blood (EDTA) Samples***

1. Pipette 80µL of each blood sample into a screw-capped microfuge tube.
2. Pipette 320µL of 170mM (9.09 g/L) NH<sub>4</sub>Cl solution into each tube.
3. Mix for 20 minutes by gentle swirling and inversion. Avoid vigorous agitation and formation of foam.
4. Centrifuge each tube for 2 minutes at 12,000g until a cell pellet is formed.
5. Using a pipette remove and discard the supernatant liquid.
6. Pipette 300µL of 10mM (0.58g/L) NaCl/10mM (3.72g/L) EDTA into each tube and resuspend the cells by vortex mixing.
7. Centrifuge each tube for 1 minute at 12,000g until a cell pellet is formed.
8. Repeat steps 5 to 7 at least a further two times until all visible red coloration in the supernatant liquid has been removed.
9. Using a pipette remove and discard the supernatant liquid.
10. Pipette 200µL of 50mM (2g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.
11. Incubate in a heated block at 100°C for 10 minutes.
12. Pipette 40µL of 1M (121.1g/L) Tris-base/HCl (pH 7.5) into each tube and vortex mix.
13. Add 1mL sterile deionised water to each microfuge tube to give a total DNA sample volume of 1.24mL.

14. Centrifuge each tube for 1 minute at 12,000g until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

### ***Preparation of DNA from Dried Bloodspots***

1. Punch 2 x 3mm discs<sup>(b)</sup> from specimen card in to a 1.5mL screw-capped tube.

**Spots should be punched from an area of card that is completely saturated with blood.**

**Punch several "clean" card spots to waste prior to each sample to prevent sample carryover.**

2. Add 1mL of 10mM (0.58g/L) NaCl/10mM (3.72g/L) EDTA and mix on rotary mixer for 15 - 20 minutes. If rotary mixer not available then washes may need to be extended up to 30 minutes each.
3. Remove and discard wash solution.
4. Repeat steps 2 and 3 once more.
5. At this point most of the haem pigment should be eluted from the disc. A faint reddish brown colouring of the blood spots is not uncommon at this stage.
6. Microfuge briefly (3 secs) to collect remaining wash solution at bottom of tube. Using a pipette, remove and discard as much wash solution as possible, without disturbing spots.
7. This brief microfuge step typically removes additional haem pigments from the blood spots.
8. Add 150µL of 50mM (2g/L) NaOH to each tube. Flick carefully with finger to mix.
9. Incubate in a heated block at 100°C for 10 minutes.
10. Microfuge briefly to collect supernatant at bottom of tube.
11. Add 30µL of 1M (121.1g/L) Tris-base/HCl (pH 7.5) into each tube, to neutralise, and mix carefully.
12. Add 420µL of sterile deionised water to each DNA sample to give a total volume of 600µL.
13. Mix samples well and microfuge to collect.
14. Transfer supernatant to a fresh labelled screw-capped tube.
15. Store extracted DNA at -20°C.

<sup>(b)</sup> Total area of blood sample used should be at least equivalent to that of 2 x 3mm circular blood spots. For example, 1x 6mm card spots may be used if convenient.

The DNA preparation method described above is recommended by Gen-Probe Life Sciences Ltd. and has been demonstrated to produce consistent and reliable results. DNA

prepared using other methods or from other sample types may not be optimal for the Elucigene AAT test and may produce sub-optimal results. The key criteria for alternative DNA preparation methods are optimal DNA concentration and absence of PCR inhibitors.

It is recommended that alternative methods and sample types are thoroughly evaluated with the Elucigene AAT test prior to the results being used for diagnostic use. Testing of DNA samples at concentrations <10ng/5µL is not recommended. Under optimal PCR conditions, results are consistently obtained at DNA concentrations between 10 and 100ng/5µL.

## Test Protocol

### Amplification Procedure

The figures given in Tables 1 and 2 can be increased proportionately for numbers of tests other than those specified. However, owing to the small volumes involved, Gen-Probe Life Sciences Ltd. recommends that no fewer than 5 tests are prepared at one time.

1. Program the thermal cycler for a time-delay file to activate the AmpliTaq Gold at 94°C for 20 minutes linked to an amplification cycling program of 30 seconds at 94°C (denaturation), 1 minute at 62°C (annealing) and 1 minute at 72°C (extension) for 35 cycles. This should be linked to a 10-minute time-delay file at 72°C (extension) on the final cycle.

**Note: Select 'Block' method option on the thermal cycler for PCR in 0.5ml vials.**

2. Thaw and centrifuge the Primer Mix A (TA), Primer Mix B (TB), AmpliTaq Gold (not provided), Loading Dye (LD) and Dilution Buffer (DB) vials for 10 seconds at 12 000g, mix gently by vortexing and centrifuge the vials again for 10 seconds.

**Note: Steps 3 - 5 must be carried out in an area free from DNA**

3. Referring to Table 1 prepare sufficient dilution of AmpliTaq Gold (not supplied) in the Dilution Buffer supplied and sterile distilled water for the number of samples and controls to be tested. Mix thoroughly by gently pipetting up and down.

Table 1. Dilution of AmpliTaq Gold in Dilution Buffer

	Number of Tests Required			
	10	20	30	40
Volume of sterile distilled water (µL)	42	84	126	168
Volume of Dilution Buffer (µL)	12	24	36	48
Volume of Loading Dye (µL)	60	120	180	240
Volume of AmpliTaq Gold (µL)	6	12	18	24
Total Volume (µL)	120	240	380	480

4. Referring to Table 2, prepare the A and B reaction mixes. Remove the appropriate aliquot of Primer Mix A into a labelled microfuge tube. Repeat with Primer Mix B into a second labelled microfuge tube. Using separate pipette tips add the appropriate volume of the AmpliTaq Gold dilution (from step 3) to each microfuge tube. Mix gently by vortexing and centrifuge the vials for 10 seconds at 12 000g.

**Table 2. Preparation of A and B Reaction Mixes**

	Number of Tests Required							
	10		20		30		40	
	A	B	A	B	A	B	A	B
Volume of Primer Mix A (µL)	165		330		495		660	
Volume of Primer Mix B (µL)		165		330		495		660
Volume of Diluted Enzyme (µL)	55	55	110	110	165	165	220	220
Total Volume (µL)	220	220	440	440	660	660	880	880

5. Label one vial 'A' and one vial 'B' for each sample and control or if coloured vials are available use a different colour for each primer mix.
6. Pipette 20µL of the prepared A reaction mix into the bottom of each of the appropriate number of the PCR vials labelled 'A'. Repeat with the B reaction mix into each of the appropriate number of the PCR vials labelled 'B'.
7. Pipette 5µL of test DNA sample or DNA Control (DC) into each of a vial A and B pair using separate pipette tips each time. Add one drop of Sigma light white mineral oil to cover the aqueous phase \*. Re-cap firmly.
8. For the negative control add no DNA to a vial A and B pair. Add 1 drop of Sigma light white mineral oil to cover the aqueous phase \*. Re-cap firmly.
9. Centrifuge the A and B vials for 10 seconds at 12 000g.
10. Place all vials firmly in the thermal cycler block. Initiate the 94°C time-delay file followed by the amplification cycling program.
11. Discard all the remaining unused AmpliTaq Gold dilution and prepared A and B reaction mixes.
12. On completion of the amplification cycling program, the samples may be stored at room temperature overnight or at 2-8°C for up to 7 days before analysis by gel electrophoresis.

\* For amplification carried out in 0.5ml PCR vials or thermal cyclers without heated lids

### **Gel Electrophoresis**

It is recommended that each user ensure that the chosen equipment is used according to the manufacturer's instructions and is compatible with this test. In this context the key parameters are the gel matrix and comb (well former) dimensions. Results have been obtained using the following electrophoresis conditions:

1. PCR product was electrophoresed in a 3% NuSieve® 3:1 agarose gel using tris-borate with ethidium bromide (TBE/EtBr) as running buffer. TBE/EtBr was prepared as 134mM (16.2g/L) Tris-base, 74.9mM (4.63g/L) boric acid, 2.55mM (0.95g/L) EDTA buffer with 0.1µg/mL ethidium bromide.
2. 3g of NuSieve® 3:1 were dissolved in 100mL TBE/EtBr and poured into a 15 x 12cm horizontal gel tray with 1.5mm x 5mm well formers suspended 1mm above the base.

3. 15 $\mu$ L of the PCR product (with the loading dye added during the PCR set-up process) were loaded onto a gel.
4. A 50 Base-Pair Ladder (GE Healthcare) was run adjacent to the samples as a molecular size marker.
5. Electrophoresis was carried out at 5 to 6 V/cm between electrodes until the dye front had migrated 4cm from the loading wells towards the anode (1 to 1.5 hours).
6. After electrophoresis the gels were placed on a UV transilluminator at 260nm then visualised and photographed.

### ***Interpretation of Results***

1. The negative control must show no bands in the A and B vial tracks within the area corresponding to 148 base pairs (bp) and 499 bp.
2. The upper and lower control bands must be clearly visible in each track corresponding to vial A and B pairs. All diagnostic product bands should be clearly visible and of a similar intensity to the control bands in that vial.
3. All tracks should be free of excessive smearing and background fluorescence.
4. The position of the upper and lower control bands should indicate the correct molecular size i.e. 499 and 148 bp

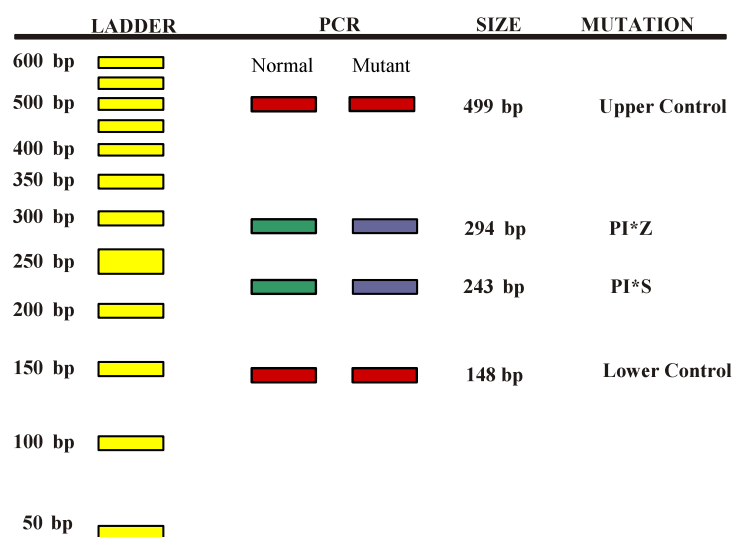
**If any of the above points are not observed the results should not be interpreted and a repeat test carried out.**

5. An individual has two copies of the AAT gene. Where these copies have the same sequence for any given site, an individual is described as being homozygous for this site e.g. PI\*ZZ. Where the copies differ in sequence at a given site, an individual is described as being heterozygous for this site.
6. PCR products from the upper and lower control primers will be observed as bands in both the A and B vial tracks of the gel at 499 bp and 148 bp respectively.
7. PCR products from an individual not carrying a PI\*S or a PI\*Z allele will be observed as bands at the positions shown in figure 1.
8. PCR products from an individual carrying a PI\*Z allele will be observed as a band in the vial B track of the gel at 294 bp. PCR products from an individual not carrying a PI\*Z allele will be observed as a band at the adjacent position in the vial A track. It follows that PCR products from a PI\*Z heterozygote will be seen as bands in both the A and B vial tracks at 294 bp.
9. PCR products from an individual carrying a PI\*S allele will be observed as a band in the vial B track of the gel at 243 bp. PCR products from an individual not carrying a PI\*S allele will be observed as a band at the adjacent position in the vial A track. It follows that PCR products from a PI\*S heterozygote will be seen as bands in both the A and B vial tracks at 243 bp.
10. It is possible to detect PI\*SZ compound heterozygotes using Elucigene AAT.

Note: PI\*ZZ samples may produce a small amount of PCR product from the primer designed to amplify the normal allele for the Z mutation site. This artefact can be observed in the vial A track of the gel when PCR conditions other than those optimised and recommended in the product literature are used.

Figure 1 shows diagrammatically the size, in base pairs, and relative location of the PCR products in a gel that is expected for the normal AAT genotype (not carrying the S or Z mutations) using the Elucigene AAT.

**Figure 1**



### Performance Characteristics

The recommended method described in this pack leaflet has been demonstrated to perform in an equivalent manner to a previously validated method using two hundred EDTA blood samples tested with the Elucigene AAT reagents in a 'blind' in-house study. Each result was confirmed for the PI\*S and PI\*Z alleles by an alternative method. The results were concordant. Of the 200 individuals tested, 179 were normal (not carrying the S or Z mutations), 15 were PI\*S heterozygote and 6 were PI\*Z heterozygote. All DNA samples amplified successfully and none required repeat testing.

### Limitations of the Procedure

1. The results obtained from this or any other diagnostic reagent should be used and interpreted only in the context of the overall clinical picture. Gen-Probe Life Sciences Ltd. is not responsible for any clinical decisions that are taken.
2. It should be noted that the genotype of a patient who has received a liver transplant for AAT deficiency will not correspond to the patient's apparent phenotype.
3. As with any genetic test, erroneous results may be obtained from blood samples that have been taken subsequent to a recent blood transfusion.
4. The absence of the two mutations detected by these reagents is no guarantee that other variants of the AAT gene are not present.

The user of these reagents should emphasise these points when reporting results to the diagnosing clinician or genetic counsellor.

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