AAT mpx RealFast™ Assay

REF 7-265 / 7-268 Σ 100 / 32 reactions

-20°C 2-8°C CE IVD

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1. Intended Use

The AAT RealFast™ Assay is a fast and accurate real-time PCR based test for the simultaneous detection of the protease inhibitor (PI) variants *S and *Z of the *SERPINA1* gene. These are the most frequent alleles associated with alpha-1 antitrypsin (AAT) deficiency. The kit is intended for use as a diagnostic tool to identify alpha-1 antitrypsin deficient patients carrying a PI*S or PI*Z allele. In a human DNA extract, the qualitative assay discriminates the possible PI genotypes: normal *MM, heterozygous *MS, *MZ or *SZ, homozygous *SS or *ZZ.
Reference sequence: NG_008290.1; HGVS: PI*S: g.14768A>T, dbSNP: rs17580; PI*Z: g.17083G>A; dbSNP: rs28929474.

2. Introduction

Alpha-1 antitrypsin deficiency is one of the most common hereditary disorders in persons of northern European heritage. The protease inhibitor alpha-1 antitrypsin (AAT) is mainly synthesized in the liver and inactivates proteases like neutrophil elastase in the lung of healthy individuals. Deficient variants are characterized by low serum levels of AAT and, for some alleles including the Z allele, also decreased enzymatic function. Unhindered proteolyse in the lungs causes damage of alveolar tissue and development of chronic obstructive pulmonary disease (i.e. emphysema, persistent airflow obstruction, and/or chronic bronchitis). Accumulation of abnormal AAT in the liver leads to liver disease, especially cirrhosis and hepatocellular carcinoma. Individuals carrying the PI*ZZ genotype are at a high risk of developing emphysema and liver disease, whereas the PI*SZ genotype is associated with a somewhat lower risk to become symptomatic. For PI*MZ individuals the situation is less clear, but accelerated lung destruction especially in smokers, has been reported. Carriers of PI*MM, *MS or *SS have normal or only slightly decreased AAT plasma levels.

3. Kit Contents

			100 / 32 Rxn
RealFast™ 2x mpx **Probe Mix**	1 vial	white cap	1000 / 320 µl
AAT mpx **Assay Mix**	1 vial	purple cap	550 / 550 µl
AAT mpx **WT-Control**	1 vial	green cap	75 / 75 µl
AAT mpx **MUT-Control**	1 vial	red cap	75 / 75 µl

The kit contains reagents for 100 / 32 reactions in a final volume of 20 µl each.

The RealFast™ 2x Probe Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The AAT mpx Assay Mix consists of *SERPINA1* gene-specific primers and four allele-specific, dual-labeled hydrolysis probes. Controls representing wild type (WT-Control) and homozygous mutant (MUT-Control) genotypes are supplied with the kit.

4. Storage and Stability

AAT mpx RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -20°C. Alternatively, store at 2 to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid prolonged exposure to intense light. If stored correctly, the kit will retain full activity until the expiration date indicated on the label.

5. Product Description

5.1. Principle of the Test

The test is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains two gene-specific primer pairs which amplify a 145 bp and a 153 bp fragment of the *SERPINA1* gene, as well as four dual-labeled, allele-specific hydrolysis probes which hybridize to the target sequences of the amplified fragments. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5' – 3' exonuclease activity of the Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product.

Hydrolysis probe	Fluorophore	Channel
PI*S mutant	FAM	520 nm
PI*M wild type	HEX	556 nm
PI*Z mutant	ROX	605 nm
PI*M wild type	Cy5	670 nm

In normal samples the **wild type probes** generate a strong fluorescence signal in the HEX or Cy5 channel and no or only a baseline signal in the FAM or ROX channel. Vice versa, in homozygous mutant samples the hybridized **mutant probes** generate a strong fluorescence signal in the FAM or ROX channel and no or only a baseline signal in the HEX or Cy5 channel. In heterozygous samples both wild type and mutant probes bind to the amplicons and generate intermediate signals in the respective channels.

5.2. Real-time PCR Instrument Compatibility

The AAT mpx RealFast™ Assay is validated for use with the AB 7500 Fast instrument.
The kit is compatible with various common real-time PCR instruments capable of recording FAM, HEX, Cy5 and ROX fluorescence:

- ✓ AB 7500 Fast (Applied Biosystems®)
- ✓ CFX96™ (Bio-Rad)
- ✓ LightCycler® 480 (Roche)
- ✓ MIC qPCR Cycler (bms)
- ✓ Rotor-Gene® 6000 (Qiagen)

» ***Note:*** *RealFast™ Genotyping QuickGuides for setting up and analyzing experiments on different types of instruments can be downloaded from www.viennalab.com.* «

The kit is **not suitable** for use with real-time PCR instruments requiring ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT).

5.3. Assay Performance Specifications

Determination of **sensitivity** was performed on 64 alleles testing positive for the PI*S and/or PI*Z allele with a CE-marked reference kit. The AAT mpx RealFast™ Assay determined all 64 alleles as positive, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 62 alleles testing negative for the PI*S or PI*Z allele with a CE-marked reference kit. The AAT mpx RealFast™ Assay determined all 62 alleles as negative, which equaled a true negative rate of 100%.

Limit of detection: 0.2 ng genomic DNA (per reaction).
Recommended DNA concentration: 2 to 20 ng/µl genomic DNA.

6. Materials Required but not Supplied

Real-time PCR instrument with FAM (520 nm), HEX (556 nm), ROX (610 nm) and Cy5 (660nm) filters, instrument-compatible reaction vessels, disposable powder-free gloves, vortexer, mini-centrifuge for 2.0 ml tubes, tube racks, set of calibrated micropipettes (0.5 – 1000 µl), sterile tips with aerosol-barrier filter, molecular grade water, DNA extraction system, freezer, biohazard waste container.

7. Experimental Protocol

7.1. DNA Extraction

DNA extraction reagents are **not supplied** with the kit.
DNA isolated from various specimens (e.g. whole peripheral blood, dried blood spots, buccal swabs or saliva) can be used. Ensure extracted DNA is suitable for amplification in terms of concentration, purity and integrity.
For accurate genotype calling, the DNA amount per reaction should be within the range of 10 to 100 ng for all samples.

7.2. PCR Controls

Always include a **No Template Control** (NTC) in each experiment to confirm absence of potential contamination. It is advisable to run the NTC (use PCR-grade water instead of DNA) in duplicate.

Always include the AAT mpx **WT-Control** and AAT mpx **MUT-Control** as positive reference signals for your unknown samples. Some real-time PCR software, e.g. AB 7500 Fast, requires signals for all three possible genotypes for correct allelic discrimination. In order to obtain a heterozygous control (HET-Control), mix an aliquot of WT-Control and MUT-Control in a ratio of 1:1.

» ***Note:*** *WT- and MUT-Controls are potential sources of contamination. Make sure to handle them carefully.* «

7.3. Preparation of AAT mpx RealFast™ **Master Mix**:

Gently vortex and briefly centrifuge all solutions after thawing. Set up PCR at room temperature. Prepare sufficient **Master Mix** for all your reactions (N samples + positive controls + negative controls) plus at least one additional reaction to compensate for pipetting inaccuracies:

Component	per reaction	e.g. 24+1 reactions
RealFast™ 2x Probe Mix	10 µl	250 µl
AAT mpx Assay Mix	5 µl	125 µl
Master Mix	**15 µl**	375 µl

Dispense **15 µl Master Mix** into each well. Add **5 µl** purified **DNA** or **Control** template to reach a final reaction volume of 20 µl.
To minimize risk of contamination, always pipette templates in the following order: first NTC, then samples, last positive controls. Immediately close reaction vessels.

» ***Note:*** *Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed.* «

7.4. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for allelic discrimination / genotyping experiments. Place the samples into the thermal cycler and run the following program:

Program			**AB 7500 Fast, CFX96™, LightCycler® 480,** and **other** Peltier heating-block based **instruments**	**MIC, Rotor-Gene® 6000** (36-well & 72-well rotor)
Cycles	Temp	Time	Steps	Steps
1	95°C	3 min	Initial denaturation	Initial denaturation
40	95°C	15 sec	Denaturation	Denaturation
	60°C	1 min	Annealing/Extension – **Data acquisition** on FAM, HEX, ROX and Cy5 channels	Annealing/Extension – **Data acquisition** on Green, Yellow, Orange and Red channels

8. Data Analysis / Interpretation of Results

The genotype of each sample is determined by calculating the ratio between signals recorded in the **HEX** or **Cy5 channel (normal)** and signals recorded in the **FAM** or **ROX channel (mutant)**. Most real-time PCR software automatically resolves data of two channels channels into clusters in a scatterplot. Data points plotted along the x- and y-axes correspond to normal and homozygous mutant genotypes, respectively. Data points clustered in the middle of the scatterplot represent heterozygous genotypes. The NTC appears in the lower left corner.

Controls / Samples	Amplification in channel				Genotype **PI* alleles**
	FAM Green	HEX Yellow	ROX Orange	Cy5 Red	
mpx WT-Control	NO	**YES**	NO	**YES**	PI*MM (normal)
mpx HET-Control	**YES**	**YES**	**YES**	**YES**	PI*SZ (heterozygous)
mpx MUT-Control	**YES**	NO	**YES**	NO	---- (positive control for PI*S and PI*Z)
NTC	NO	NO	NO	NO	----
Sample 1	**YES**	**YES**	NO	**YES**	PI*MS (heterozygous)
Sample 2	**YES**	NO	NO	**YES**	PI*SS (homozygous)
Sample 3	NO	**YES**	**YES**	**YES**	PI*MZ (heterozygous)
Sample 4	NO	**YES**	**YES**	NO	PI*ZZ (homozygous)

Some instrument software needs manual threshold settings for accurate genotype calling.

Recommendations for Threshold Settings (C_q):
Set threshold value for the FAM and ROX channels just above the background fluorescent signal generated by the WT-Control (HEX-/Cy5-positive). Vice versa, set threshold value for the HEX and Cy5 channels just above the background fluorescent signal of the MUT-Control (FAM-/ROX-positive).
Samples crossing the threshold line beyond C_q 37 give invalid results and must be repeated.

To analyze acquired data, please follow your instrument software instructions.

9. Warnings and Precautions

- For *in vitro* diagnostics use only.
- Always use disposable powder-free gloves and wear suitable lab coat when handling specimens and reagents.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use pipettes dedicated for PCR setup only, use aerosol-guarded pipette tips.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.
- Do not use expired kits or kit components.