LightMix® in-vitro diagnostics kit

*Alpha1-Antitrypsin (AAT) Pi*S and Pi*Z*

Cat.-No.: 40-0576-64

Detection of the Pi*S and Pi*Z
DNA variations in the AAT gene

for use with the

Roche Diagnostics LightCycler® Instruments

Reagents for 64 reactions

Upon arrival:

Store Premixed PCR reagents and Controls
protected from light at room temperature or cooled (do not freeze)

Store FastStart DNA Master HybProbe reagents frozen (-20°C)
(if included)

CE IVD

Manual version V160626

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1. Product Information

1.1 Contents: LightMix® Kit AAT Pi*S and Pi*Z

Lyophilized premixed PCR reagents

⚠ **Store cooled or at room temperature(4-25°C) in the dark**

	Cap color	Label	Description content	Reaction / Tube status	Total
1 x	Red	PSR	Parameter Specific Reagents (PSR) containing premixed and lyophilized primers and probes for 64 reactions. <0,01pg unlabeled oligonucleotides (AAT Pi*S and Pi*Z primers); <0,01pg SimpleProbe® 519 labeled oligonucleotides (AAT Pi*S); <0,01pg LightCycler-Red640 labeled oligonucleotide (AAT Pi*Z Probe); <0,01pg Fluorescine 0 labeled oligonucleotide (AAT Pi*Z Anchor Probe)	64reactions lyophilized	64 rxs

Standards (Control DNA)

⚠ **Store cooled or at room temperature (4-25°C) in the dark**

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	Yellow	AAT Pos	Positive Control AAT Pi*S and Pi*Z and compound heterozygous <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	40 reactions lyophilized	40 rxs
1 x	Yellow	AAT Pi*S	Genotyping Standard AAT Pi*S Homozygous mutant (channel 530 mutant, 640 wild type) <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	40 reactions lyophilized	40 rxs
1 x	Yellow	AAT Pi*Z	Genotyping Standard AAT Pi*Z Homozygous mutant (channel 530 wild type, 640 mutant) <0,01pg plasmid target (synthetic) [about 10E4 genome equivalents]	40 reactions lyophilized	40 rxs

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ **Store at -20°C upon arrival**

FastStart DNA Master HybProbe is included in kits supplied directly by TIB MOLBIOL for customers in Central Europe only (1).
The FastStart DNA Master HybProbe is not included in AAT kits supplied through Roche Diagnostics or its local distributor.

	Cap color	Label	Description content	Reaction Tube storage	Total
1 x	Red	1a	LightCycler® FastStart Enzyme	64 reactions frozen	64 rxs
1 x	White	1b	LightCycler® FastStart Reaction Mix Hyb-Probe	64 reactions frozen	64 rxs
1 x	Colorless	Water	H_2O PCR grade	frozen	64 rxs
1 x	Blue	$MgCl_2$	$MgCl_2$, 25 mM	frozen	64 rxs

1) FastStart enzyme is shipped by TIB MOLBIOL at ambient temperature.

1.2 Intended Use

This kit allows the detection of mutations in codons 264 (Pi*S) and 342 (Pi*Z) in the Alpha-Antitrypsin (AAT) gene, also known as Protease Inhibitor I (Pi) (OMIM 107400) in genomic human DNA from a nucleic acid extract obtained from peripheral blood.

AAT-deficiency is linked in particular to mutations in codons 264 and 342. Other SNP variants detected with this kit are not known to be associated with AAT-deficiency. This kit gives an indication on the presence of a deletion of the AAT gene (Pi*00).

This kit is intended to help the clinician to analyze the genetical background for the risk of chronic obstructive pulmonary disease (COPD), Emphysema, and liver cirrhosis.

The present test can be performed in addition or after a biochemical assay measuring AAT serum levels.

Results obtained using this kit are not intended to be the only basis for any therapy decision. Patient´s mutation status should be considered alongside other disease factors.

Note: The performance of the assay can be guaranteed only when used with LightCycler® Instruments (see 1.3.2 for details).

The present product is an *in-vitro* diagnostic device, which must be used by qualified personnel only.

1.3 Specifications

The *LightMix® Kit AAT Pi*S and Pi*Z* is an *in-vitro* diagnostic test which allows the detection of clinical relevant single nucleotide polymorphisms (SNP) in the Alpha1-Antitrypsin (AAT) gene known as Pi*S and Pi*Z variants.

Other variants also detected with this kit are listed in section 7.6.

1.3.1 Clinical Samples

The test requires 2 µl of purified genomic DNA in aqueous solution extracted from clinical specimen, containing from 5 to 100 ng/µl of genomic DNA (10 ng – 200 ng total amount), as determined by UV spectrophotometry (1 OD = 50 µg DNA/ml).

1.3.2 Instruments, software and productivity

One kit contains reagents for 64 reactions performed in a 10 µl volume.
Each run requires including one standard and one negative control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Run Time (approx.)	Max Samples per run (2)	Maximum Productivity of the kit (3)	Minimum Productivity of the kit (4)
LC 1.2	4.10 (1)	60 min	30 + 2 ctrl.	58	20
LC 1.5	4.10 (1)	60 min	30 + 2 ctrl.	58	20
LC 2.0	4.05	60 min	30 + 2 ctrl.	58	20
LC480 (96 wells)	1.5	100 min	94 + 2 ctrl.	60	20
LC480 (384 wells)	1.5	100 min	382(5) + 2 ctrl.	60	20
Z480 (open channel)	1.5	100 min	94 + 2 ctrl.	60	20
Nano	1.0 (6)	60 min	30 + 2 ctrl.	60	21

1 Running the test with the LightCycler® 1.2 or 1.5 Instruments with the software version 3.5 yields comparable results. Instruction for programming, data analysis and interpretation of results are not described in this manual. **Upgrade to version 4.10 or higher when possible**. LightCycler® software 3.5.3 does not contain the automatic genotyping module; equivalent results can be obtained by trained personnel which must analyze each sample manually.
2 Each run must include 1 standards and one No-Target Control (NTC) for a total of 2 control reactions.
3 The first run of the kit requires including 4 controls (instead of 2) to teach the genotyping module. The maximum number of samples that can be processed is reduced accordingly. Depending on local regulations, all 4 genotyping controls may have to be included in each run, reducing the total number of patient's samples that can be analyzed.
4 Calculated considering a single clinical sample analyzed in each run.
5 It requires using six kits.

6 Nano LightCycler® software 1.0 does not contain the automatic genotyping module, therefore it is not necessary to add the Genotyping Standards; equivalent results can be obtained by trained personnel which must analyze each sample manually.

1.4 Storage and Stability

Note the **different storage conditions** for reagents and polymerase mix!

Storage Conditions

Reagents and Controls:

Store the lyophilized reagents (PSR and Standards) protected from light and at room temperature or cooled (4°C - 25°C).
Do not freeze these dried reagents. Expiration date is printed on the kit label.

Polymerase mix :

Store the LightCycler® FastStart DNA Master HybProbe at -15°C to -25°C.
See expiration date on the polymerase tube label.

Shipping:

Products are shipped at ambient temperature. Transport stability of reagents and enzyme components have been tested under shipping conditions.

2. Additional Devices and Reagents

2.1 Required

	TIB Molbiol
LightMix® Kit – Color Compensation 530-640-690 (not required for LightCycler® Nano Instrument)	Cat.-No. 40-0318-00
LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Cat.-No. 12 011 468 001
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
Or	
LightCycler® 480 Instruments	**Roche Diagnostics**
LightCycler® 480 Instrument (model I)	Discontinued
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
Cobas® 4800 System (Z480 Instrument)	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
Or	
LightCycler® Nano Instrument	**Roche Diagnostics**
LightCycler® Nano Instrument	Cat.-No. 06 407 773 001
LightCycler® Software Version 1.0 or higher	Included with Instrument
LightCycler® Nano tubes	Cat.-No. 06 327 672 001
Or	
LightCycler® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001

2.2 Optional

Instruments:

LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.3 Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Nuclease-free PCR grade water	any supplier
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier
Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure 2.0 Instrument	Cat.-No. 05 197 686 001
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument	Cat.-No. 03 731 146 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
MagNA Pure 96 Instrument	Cat.-No. 05 195 322 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 05 467 497 001
MagNA Pure 96 IVD Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001

3. Background Information

3.1 Medical Background

Alpha 1-antitrypsin deficiency (A1AD) was discovered 1963 by C.B. Laurell by protein analysis of patients with emphysema onset at a young age [1].

Alpha-1 antitrypsin is a protease inhibitor belonging to the serpin superfamily. The formal name is Serpin Peptidase Inhibitor (SERPINA1). In the past the protein has been also referred to alpha-1 proteinase inhibitor (A1PI).

Today we know that A1AD is a genetic disorder causing production of defective enzyme with decreased A1AT activity in the blood and lungs, and deposition of excessive abnormal A1AT protein in the liver, caused by missing AAT enzyme in case of a deletion of the gene.

A1AD causes emphysema, a long-term progressive disease of the lungs that primarily causes breath shortening, or chronic obstructive pulmonary disease [2] (COPD), and less frequent damages of the liver. It is treated by avoidance of damaging inhalants - carriers should quit smoking – and, in severe cases, by infusions of the AAT protein or even transplantation of liver or lungs. A1AD reduces the life expectancy. For review see Stoller and Aboussouan, 2005 [3].

Carriers of two defect alleles are affected, with a significant higher morbidity related to the Pi*Z mutation (p.E342K substitution glutamate 342 by lysine) than for Pi*S (p.E264V substitution glutamate 264 to valine). Many other AAT mutations located near amino acid 342 have been described, but there are no reports about a relation to A1AD. Deletion of the gene (allele Pi*00) also causes A1AD, but without a damage of the liver.

Nomenclature for 342 and 264 AAT mutations (COSMIC and NCBI):

Amino Acid	Allele	cDNA Position	Genomic Position	dbSNP
p.E342K	Pi*Z	c.1024G>A	g.11940G>A	rs28929474
p.E264V	PiS	c.791A>T	g.9628T>A	rs17580

The frequencies for the Pi*Z and Pi*S alleles are in the range of 1.5% and 3%, respectively. The prevalence for A1AD is estimates to be in the range from 1:1.600 to 1:5,000. The American Association for Clinical Chemistry reports a prevalence of 1:3,000.

The initial diagnosis is based on serum A1AT levels, followed by AAT protein phenotyping and/or genotyping [4]. Analysis of the AAT mutation based on melting curves have been published already 1999 by von Aslanidis et al.[5].

3.2 Methodology and Assay Principle

Using PCR methodology, two fragments of the AAT gene are simultaneously amplified with specific oligonucleotide primers. Fluorescent labeled probes are used to identify the PCR product and to determine the genotype by performing a melting curve analysis.

The probe binds to a part of the amplified fragment spanning the mutation site. Any mismatch covered by the probe destabilizes the hybrid. During the melting curve analysis the temperature is slowly increased. The probe melts off at a specific melting temperatures causing the fluorescence to decrease.

Pi*Z allele (p.E342K)
A 229 bp long PCR fragment containing the G11940A (rs28929474) site is analyzed using a LightCycler® Red 640 labeled SimpleProbe® oligomer which is matching the rare 11937A variant (SNP rs143370956). In the melting curve analysis the 11937A samples display a higher temperature than the wild type allele, while the Pi*Z risk allele 11940A has the lowest melting temperature. Other variants yield intermediate melting temperatures (see 7.6).

Pi*S allele (p.E264V)
The second PCR product has a length of 177 bases and includes the T9628A (rs17580) polymorphic site and is analyzed with a 519 labeled SimpleProbe® oligomer identical to the 9628A allele, resulting in a high melting temperature for the Pi*S variant and a lower melting temperature for the wild type allele.

Pi*00 allele (gene deletion)
In case of a AAT gene deletion there will be no melting peaks visible, but the result can not be distinguished from missing or low amount of sample DNA or inhibition of amplification or other PCR failures. For samples reporting no melting peaks the test should be repeated and verified with a second method.

Reading of the genotype results is based on the melting temperatures compared to the supplied standards. If permitted by the instrument software reading of the genotype results can be achieved by the automated genotyping (instrument-dependent: software module 'Melt Curve Genotyping').

Automated genotyping results must be reviewed by eye for deviating curves and intermediate melting point temperatures. In case that automated typing fails to report consistent genotype results, the genotype must be deducted from the melting temperatures following the criteria described in chapter 7. See also section 7.6 for melting temperatures expected for other variants.

The kit contains DNA standards for the Pi*S, Pi*Z and the wild type alleles to enable a comparison with clinical samples.

3.3 Performance Characteristics

Analytical Specificity

The specificity to the target gene and the suitability of the PCR amplification employed in the present kit was demonstrated by comparison with the results obtained by direct sequencing.

Analytical Sensitivity

Detection of serial dilutions of several heterozygous human genomic DNAs has revealed that the limit of detection of the present kit is 250 copies (1.5 ng).

Diagnostic Specificity and Sensitivity

A total number of 98 genomic DNA samples from Caucasian origin individuals were analyzed in comparison with another AAT typing kit which is known to use different primer and probe sequences; in addition, 2 reference samples EV/EE, 4 samples EE/EK and 2 samples EE/KK were included.

Both assay showed 100% concordance. In particular, 98 samples were EE/EE and 2 samples were EV/EE. The reference samples showed the expected genotype.

In addition, 8 samples variant EE/EE, 4 samples EE/EK, 2 samples EV/EE, and 2 samples EE/KK, were confirmed by DNA sequencing.

Other variants have not been observed.

Study results: Results for both analytical methods were in 100% concordance. Diagnostic Sensitivity was 100%, Diagnostic Specificity was 100%.

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.

Use the manual version which is delivered with the kit (see kit label).

Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product employed (see chapter 2.3).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual .

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "PCR protocol" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Programming

5.1 Color Compensation

Color Compensation is required for the use of the
*LightMix® Kit AAT Pi*S and Pi*Z.*
Analyze data with 'Color Compensation'.
Its deactivation will generate invalid readouts of the results.
Color Compensation is not required for the LightCycler® Nano.

5.2 Capillary Based LightCycler® Instruments

For details see the LightCycler® Operator's Manual.
The protocol consists of four program steps (Tab.1):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	43	75	40
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate [°C/s]*	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [°C]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Size [°C]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

* For LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' [°C/s] instead of Ramp Rate.

Tab. 1:
Programming of capillary based Instruments for using *LightMix® Kit* AAT Pi*S and Pi*Z
Note:
While programming maintain default software values: channel = 530, max. samples = 32, seek temperature = 30°C and capillary size = 20 µl. Do not change the capillary size value to 100 µl.
Store the program and the default values as '**RUN Template**' which can be loaded to start every AAT LightCycler® run.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.

5.3 LightCycler® 480 Instruments

For details see the LightCycler® Operator's Manual.

Detection Format: TIB MOLBIOL 530-640
Please refer to the manual of:
LightMix® Kit- Universal HybProbe Color Compensation.
Cat. No. 40-0318-00

Reaction Volume: 10 µl

Programming:
The protocol consists of four program steps (Tab.2):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	43	75	40
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold [hh:mm:ss]	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate [C°/ s] **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.29**	**1.5**
Ramp Rate [C°/ s] **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.29**	**2.0**
Acquisitions [per °C]	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**
Sec Target [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size [°C]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 2: Programming of LightCycler® 480 Instruments (96 well and 384 well formats) and Z480 Instrument for using the *LightMix® Kit* AAT Pi*S and Pi*Z

Note:

a) Store the program and the default values as '**RUN Template'** which can be loaded to start every AAT LightCycler® run.
b) Ensure to program only 1 acquisition per second instead the default value 3; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.

5.4 LightCycler® Nano Instrument

For details see the LightCycler® Operator's Manual.

Color Compensation is not required!

Run Setting / Optical setting

Intercalating Dyes
Normal Quality

Profile

The temperature profile consists of four program steps (Tab.3):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Denaturation** of amplified PCR product.
4. **Melting** Identification of PCR amplified DNA sequence

Step:	**1**	**2**			**3**	**4**	
Parameter							
Name	**Hold**	**3 Step Amplification**			**Hold**	**Melting Stage**	
						Initial Stage	**Final Stage**
Cycles		**45**					
Temp [°C]	**95**	**95**	**60**	**72**	**95**	**43**	**75**
Ramp (°C/s)	**5**	**5**	**4**	**5**	**5**	**4**	**0.2**
Hold (s)	**600**	**10**	**15**	**20**	**30**	**120**	**1**
Acquire			√				

Tab. 3: Programming of LightCycler® Nano Instrument for using the *LightMix® Kit AAT Pi*S and Pi*Z*

Note:

Store the program and the default values as **'Experiment file'** which can be loaded to start every AAT LightCycler® run.

6. Experimental protocol

Program the Instrument before preparing the solutions (see 5. Programming and read the LightCycler® Operator's Manual for details).
The described performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® systems.

6.1 Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin is strongly discouraged since this anticoagulant might interfere with the PCR.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instruments using the extraction kit appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

In the depicted assays (7.5. Reading of the Results) the DNA was manually extracted from 200 µl of blood using the High Pure PCR Template Preparation Kit following the manufacturer's instructions; 100 µl of elution buffer were used for the final elution of the purified DNA from the column.

6.2 Reagents Preparation

6.2.1 Preparation of the LightCycler® FastStart DNA Master

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30°- 35°C for 3 - 5 minutes.
3	Quickly spin tubes to collect drops.
4	The solution must be free of particles.
5	Add 60 µl of **1b** to the vial **1a**.
6 ⚠	Mix the solution carefully with a pipette. Do not vortex! Avoid the production of bubbles.
7	Spin the tubes to collect drops.
8	Use reagent to prepare the Reaction Mix (6.3).
9	Store left over reagent at 4°C.

6.2.2 Preparation of Parameter-Specific Reagents (PSR)

►	Each **PSR** reagent tube is sufficient for 64 reactions.
1	Spin the premixed **PSR** tube at 10,000 RPM for 1 minute.
2	Check that the pellet is located at the bottom.
3	To each **PSR** tube add **66 µl** of PCR-grade **Water.**
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 1 µl of **PSR** reagent for a 10 µl PCR reaction.

6.2.3 Preparation of Positive Control

►	**Positive Control** reagent tube is sufficient for 40 reactions.
1	Spin the tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 80 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **2 µl** of each **Standard** for a 10 µl PCR reaction.
► **Positive Control** must be used in each run.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.2.4 Preparation of Genotyping Standards

The LightCycler® software 4.05 and later (capillary based instruments) and software 1.5 (LightCycler®480 instruments) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples.

►	Each **Genotyping Standard** reagent tube is sufficient for 40 reactions.
1	Spin the tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 80 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **2 µl** of each **Genotyping Standard** for a 10 µl PCR reaction.
► Both **Genotyping Standards** must be used in the first run of the kit to calibrate the genotyping module.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.3 Preparation of the Reaction Mix

6.3.1 Preparation of 64 LightCycler® Reaction Mix

We recommend preparing 64 reactions to prevent storage of dissolved or activated reagents in varying volumes (6.2). See chapter 6.4 for storage and stability of dilute components.For the preparation of reaction mix for less reactions, please go to step 6.3.2 “Reaction mix for single reaction”.

Prepare the reaction mix in the PSR reagent tube (cooled):

Components	**64 reactions**
To the **PSR** tube (red cap) already containing	66.0 µl
Add:	
H_2O, PCR-grade (colorless cap)	343.2 µl
Mg^{2+} solution 25 mM (blue cap)	52.8 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	66.0 µl
 Substitute of the “long neck cap” of the **PSR** tube with the red cap from FastStart	
Total Volume	**528.0 µl**

Tab. 4: Volumes of components for preparing 64 reaction mixture

6.3.2 Preparation of the Single LightCycler® Reaction Mix

Prepare the reaction mix by multiplying each volume (Tab. 5) by the number of biological samples to be analyzed plus three reactions (Negative Control, **Positive Control**, one excess) and (optionally) two **Genotyping Standards**.

Prepare the reaction mix in a cooled vial:

Components	**Single reaction**
H_2O, PCR-grade (colorless cap)	5.2 µl
Mg^{2+} solution 25 mM (blue cap)	0.8 µl
PSR (red cap), see **6.2.2**	1.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap), see **6.2.1**	1.0 µl
Volume of reaction mix	**8.0 µl**

Tab. 5: Volumes of components for preparing a single reaction mixture

6.3.3 Capillary / Well Loading Procedure

Each run must include one Negative Control (**NTC**) to demonstrate the absence of contaminations with genomic DNA or AAT PCR product and the **Positive Control** to identify run specific melting temperatures. Regulatory agencies or local laboratory rules might require including also the two Genotyping Standards.

Capillary / Well Loading Procedure

▶	Remember to include the controls when setting up the run.
1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **8 µl** per capillary/well of reaction mix
3	**Mandatory:** Add **2 µl** of PCR-grade **H_2O** as **Negative Control** (**NTC**) in position 1 (A1). Add **2 µl** of **AAT Pos** in position 2 (A2).
	Optional*: Add **2 µl** of **AAT Pi*S** in position 3 (A3). Add **2 µl** of **AAT Pi*Z** in position 4 (A4).
4	Add **2 µl** of **Sample** in the remaining capillaries / wells.
5 ⚠	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the LightCycler® Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment's name when instructed.
10	Save sample data in the samples' window.

* See section **6.5** for the Sample loading and Genotyping Standards calibration.

6.4 Storage and Stability of Diluted Components

Reaction Mix
The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (4°C - 8°C) for 30 days.

Avoid prolonged exposure to light.

Parameter Specific Reagents (PSR)
Once diluted, store PSR refrigerated at 4°C - 8°C for up to 30 days.

Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe
The combined FastStart DNA Master HybProbe master mix (1a+1b) can be stored refrigerated (4°C - 8°C) for 30 days.

Positive Control
The dissolved **Positive Control** is stable for 30 days when stored refrigerated (4°C - 8°C).

Genotyping Standards
The dissolved **Genotyping Standard** is stable for 30 days when stored refrigerated (4°C - 8°C).

6.5 Loading of Controls and Genotyping Standards

Samples in positions 1 and 2 (plate: A1 and A2) must be filled in each run as described in the table below.

⚠ Genotype results are based on melting temperatures.
The use of the automated genotyping module present in the LightCycler® 2.0 and LightCycler® 480 software is optional.
Genotyping Standards in positions 3 and 4 (plate: A3 and A4) are required for teaching the genotyping module only in the first run of the kit.

Refer to LightCycler® Operator's Manual for details.

6.5.1 Capillary Based Instruments

In "Samples data - Capillary View", input Sample Name as described in the second column.
Select "Analysis Type – Genotyping". Select Channel 530 and 640 only!
From the pull down menu select "Sample Type" and copy the "Genotype" description.

Pos	Sample Name	Channel	Target Name	Sample Type	Genotype (amino acids)
1	**NTC**	530	Target 1	Negative Control	
		640	Target 4	Negative Control	
2	**AAT Pos**	530	Target 1	Melting Standard	264 EV Pi*S Heterozygous
		640	Target 4	Melting Standard	342 EK Pi*Z Heterozygous
3	**AAT Pi*S**	530	Target 1	Melting Standard	264 VV Pi*S Mutant
		640	Target 4	Melting Standard	342 EE Pi*Z Wild Type
4	**AAT Pi*Z**	530	Target 1	Melting Standard	264 EE Pi*S Wild Type
		640	Target 4	Melting Standard	342 KK Pi*Z Mutant

6.5.2 LightCycler® 480 Instruments

In the "Sample Editor" window, in "Step1: Select Workflow" section, select "Melt Geno". Input the description of **Positive Control** and **Genotyping Standards** as follows:

Pos	Filter combination	Sample Name	Melt Geno Sample Type	Melt Geno Genotype
A1	Fluos (465-510)	**NTC**	Negative Control	
A1	Red 640 (498-640)	**NTC**	Negative Control	
A2	Fluos (465-510)	**AAT Pos**	Melting Standard	264 EV Pi*S Heterozygous
A2	Red 640 (498-640)	**AAT Pos**	Melting Standard	342 EK Pi*Z Heterozygous
A3	Fluos (465-510)	**AAT Pi*S**	Melting Standard	264 VV Pi*S Mutant
A3	Red 640 (498-640)	**AAT Pi*S**	Melting Standard	342 EE Pi*Z Wild Type
A4	Fluos (465-510)	**AAT Pi*Z**	Melting Standard	264 EE Pi*S Wild Type
A4	Red 640 (498-640)	**AAT Pi*Z**	Melting Standard	342 KK Pi*Z Mutant

6.5.3 LightCycler® Nano Instruments

Samples:

Input, as shown below, the description of **Positive Control** and optionally **Genotyping Standards** into the “Samples” window; input name and select Dye into the “Target” window.

Samples:

Color	Name	Note
	NTC	
	AAT Pos	
	AAT Pi*S	
	AAT Pi*Z	

Target:

Color	Name	Dye	Reference
	channel 530	FAM	
	channel 640	Cy5	

Well as table

Pos	#	Note	Sample	Cy5	Type	FAM	Type
A1	1		**NTC**	channel 640	U	channel 530	U
A2	2		**AAT Pos**	channel 640	U	channel 530	U
A3	3		**AAT Pi*S**	channel 640	U	channel 530	U
A4	4		**AAT Pi*Z**	channel 640	U	channel 530	U

7. Data Analysis and Interpretation

7.1 Limits and Interferences

The present assay is specific for the AAT gene and enables to detect common variants in codons 264E/V (allele Pi*S) and 342E/K (allele Pi*Z).
The test gives hints on the null allele (Pi 00), but missing melting peaks alone are not a strict evidence for presence of this genotype.

7.2 Calibration

Calibration has to be performed following the procedure described in 6.2.4, 6.3.3, 6.5, 7.3.2 and 7.3.3.

7.3 Quality Control – Acceptance Criteria

In order to perform a reliable genotyping analysis, it is essential that Negative Control **NTC** and **Positive Control** are included in each run.

NOTE: The test is performed at an annealing temperature of 60ºC at which the probes will not bind well, yielding low or even no signals in 'quantification'. For this reason, the acceptance criteria are based only on the definition of the melting-curve patterns as described below.

7.3.1 Negative Control

NTC Negative Control (Mandatory - position 1).
Melting-curve analysis of the Negative Control must provide a negative result: no assay-specific melting peaks (see 7.3.2) must be detected.

In case that the **NTC** should report one or more specific peaks (compare signal with sample results to avoid that the software enlarges background noise to window size suggesting the presence of melting peaks), a contamination or a pipetting error has occurred; the session is not valid and the procedure has to be repeated. If the problem sustains, change water and/or reagents and repeat. Feel free to ask for assistance at service@tib-molbiol.de.

In case a peak is detected at an unspecific temperature (see paragraph 7.3.4), the software might incorrectly identify it as positive, causing automatic genotyping impossible (LightCycler® 480 software 1.5 reports : "*Sample NTC in position A1 is a negative control not in the negative group"*).

In this case - to enable the automatic genotyping – change the NTC sample from "Negative Control" to "Unknown" (see 6.5 Sample loading and calibration of Genotyping Standards); alternatively, results must be read from the melting temperatures (see 7.3.4 Samples and 7.7 Interpretation of the Results).

7.3.2 Positive Control

Melting-curve analysis should always show
AAT Pos Positive control (Mandatory - position 2):
two melting peaks in channel 530 at 50°C±2.5°C and 56°C±2.5°C
two melting peaks in channel 640 at 55°C±2.5°C and 62°C±2.5°C

Positive Control mimic compound **heterozygous** clinical samples (see **7.5**).

7.3.3 Genotyping Standards

Melting-curve analysis should always show

AAT Pi*S Genotyping Standard (Optional - position 3):
one melting peaks in channel 530 at 56°C±2.5°C
one melting peaks in channel 640 at 62°C±2.5°C
AAT Pi*Z Genotyping Standard (Optional - position 4):
one melting peaks in channel 530 at 50°C±2.5°C
one melting peaks in channel 640 at 55°C±2.5°C

Genotyping Standards mimic homozygous mutant clinical samples (see **7.5**).

7.3.4 Samples

The result of the present assay must always show one or two melting peaks for each channel in any of the possible combinations:
in channel 530 at 50°C±2,5°C / 55°C±2.5°C
in channel 640 at 55°C±2,5°C /62°C±2.5°C
⚠ No more than two peaks per sample are expected in channel 640.
The melting peak profiles must be conformable to the acceptance criteria described in this chapter. Otherwise, the result is not valid and the procedure has to be repeated (sample preparation, amplification and detection).
See also **7.7 Interpretation of results** and **7.6 Rare Variants**.

7.3.5 Deviating Melting Curves

There are several neighboured gene variations covered by the probes used in this device. Sample results for dbSNP listed SNP are depicted in section 7.6.
Please report any deviations to service@tib-molbiol.de

Feel free to send deviant melting samples to the Berlin laboratories to confirm the obtained results and/or identify other mutations by DNA sequencing.

7.3.6 Missing peaks in both channels

Missing peaks can be due to low amount of DNA in the sample or inhibition, or the very rare case of the deletion of the AAT gene (Pi00 allele), which is associated with AAT deficiency (high risk). We strongly recommend to submit such samples for further analysis to a specialized laboratory.

7.4 Saving External Genotyping Standards

⚠ (Not applicable for LC1.x software versions below 4.0 and for LightCycler® Nano Instrument).
After the genotyping analysis, if samples 1 to 4 comply with the acceptance criteria (see **7.3 Quality Control – Acceptance Criteria**), save the Genotyping Standards as follows and use External Standard in all successive runs.

7.4.1 Capillary Based Instruments

In the Melting Curve analysis - Genotyping window open the “Standard (Int)” menu and select “Save standards as External”.

7.4.2 LightCycler®480 Instruments

In the Melt Curve Genotyping analysis window open the “Standards (In-run)” menu and select “Save as ext.”

7.5 Reading the Results

Perform data analysis as described in the LightCycler® Operator's Manuals.

7.5.1 Typical Data for Amplification

The amplification curves do not contain any analytical information (please see section **7.3 Quality Control – Acceptance Criteria**), but, nevertheless, an example from LightCycler® 2.0 is depicted below (Fig. 1 and 2).

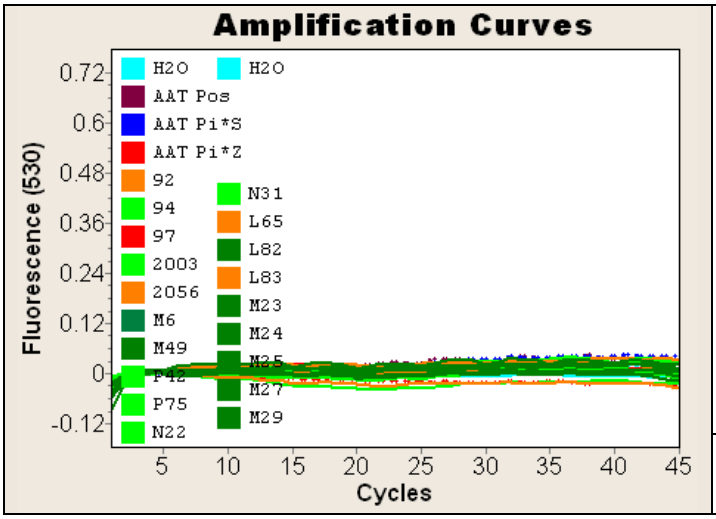
View data for amplification as follows:

LC 2.0 Instrument (or LC1.x with software versions 4.1):
View AAT Pi*S amplification in channel 530 and AAT Pi*Z amplification in channel 640, "Absolute Quantification" analysis mode.

LC 480 Instruments:
For use in LightCycler® 480 Instrument, view AAT Pi*S amplification in channel 483-533 and AAT Pi*Z in channel 483-640, "Abs Quant/2nd Derivative Max" mode.
For use in LightCycler® 480 II Instrument, view AAT Pi*S amplification in channel 465-510 and AAT Pi*Z in channel 498-640 "Abs Quant/2nd Derivative Max" mode.

LC Nano Instrument:
View AAT Pi*S amplification in "Automatic Quantification" mode in channel 530 and AAT Pi*Z in channel 640.

LC1.x, software versions 3.5:
View AAT Pi*S amplification in fluorescence channel F1 and AAT Pi*Z amplification in channel F2, "Quantification – Second Derivative Maximum" mode.

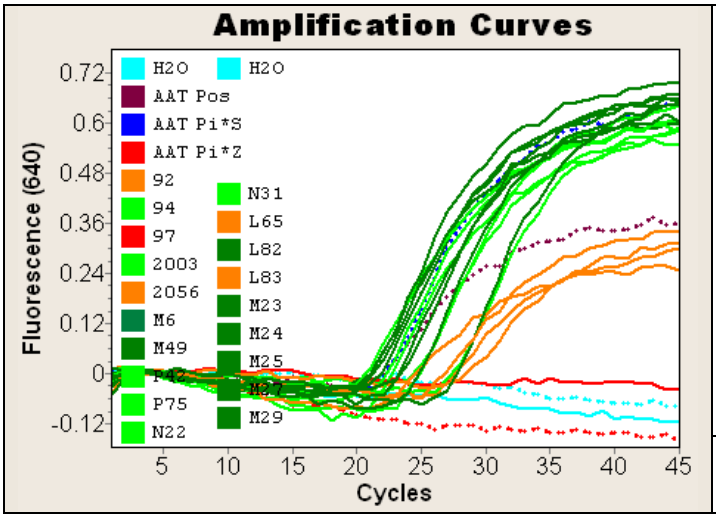


Data from channel 530

The **NTC** Negative Control (light blue line) shows no amplification signal.

All standards and biological samples show no amplification signal.

Fig. 1: Amplification plot.
Sample data for AAT Pi*S



Data from channel 640
The **NTC** Negative Control (light blue line) shows no amplification signal.

AAT Pi*Z Genotyping Standard (red dotted line) and biological sample **97 show no amplification signal.**

All other remaining standards and biological samples shows an amplification signal typical for clinical samples that contain at least one wild type allele.

Fig. 2: Amplification plot.
Sample data for AAT Pi*Z

7.5.2 Melting Analysis: Capillary Based Instruments

The melting-curve peaks (Fig. 3 and 4) display all genotypes.

Activate Color Compensation!
Analyzing data without 'Color Compensation' (deactivated) will generate invalid readouts of the results.

View AAT data for Melting as follows:
LC 2.0 Instrument (or LC1.x with software versions 4.1):
View Melting data for AAT Pi*S in channel 530 (Figure 3) and AAT Pi*Z in channel 640 (Figure 4). Analysis Type “Melting Curve Analysis – Genotyping” mode.
LC1.x, software version 3.5.3
View Melting data for AAT Pi*S in channel F1 instead of channel 530 (Figure 3) and AAT Pi*Z in channel F2 instead of channel 640 (Figure 4). “Melting Curve” mode.

Channel 530

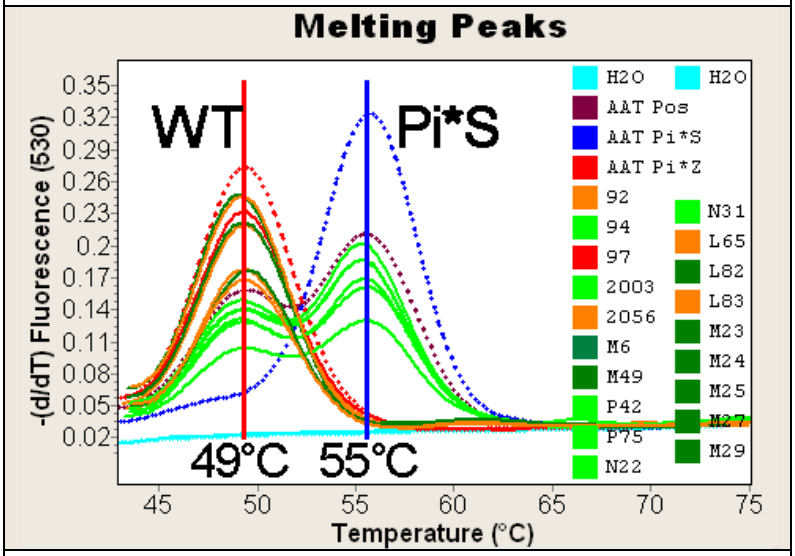


Fig. 3: Melting sample data for Kit AAT Pi*S.

NTC Negative Control
(light blue line – position 1)
no assay-specific melting peaks must be detected.
AAT Pos Positive Control
(burgundy dotted line – position 2) shows a melting peak at 49°C and a melting peak at 55°C as Pi*S heterozygous samples (E264V) (light green lines).
AAT Pi*S Genotyping Standard
(blue dotted line – position 3)
shows a melting peak at 55°C mimicking Pi*S mutant samples (264V).
Pi*Z Standard (red dotted line - position 4) shows a melting peak at 49°C as Pi*S wild type samples (E264) (dark green, red and orange lines).

Channel 640

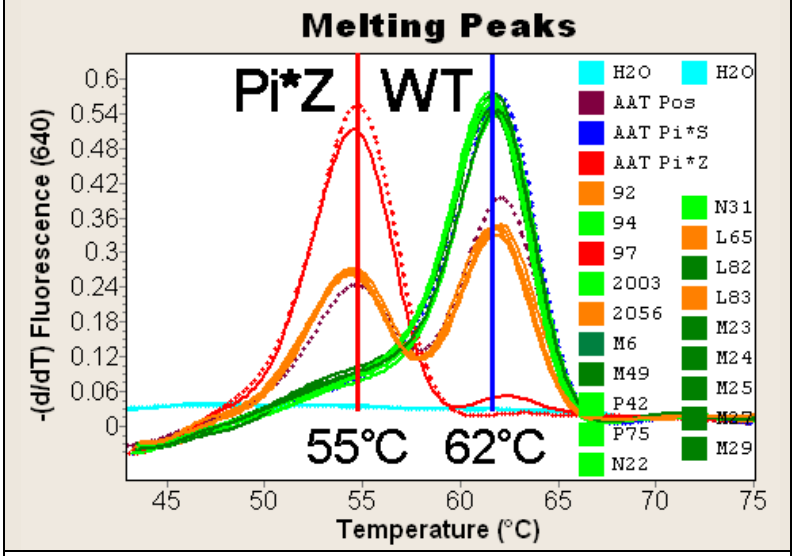


Fig. 4: Melting sample data for Kit AAT Pi*Z.

NTC Negative Control
(light blue line–position 1)
no assay-specific melting peaks must be detected.
AAT Pos Positive Control (burgundy dotted line – position 2) shows a melting peak at 55°C and a melting peak at 62°C as Pi*Z heterozygous samples (E342K) (orange lines).
AAT Pi*S Genotyping Standard
(blue dotted line – position 3) shows a melting peak at 62°C for Pi*Z wild type samples (E342) (green lines).

AAT Pi*Z Genotyping Standard (red dotted line – position 4) shows a melting peak at 55°C as Pi*Z mutant samples (342K) (red lines).

Note: The values of the melting temperatures may vary ±2.5°C between different experiments. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions: **7.3.5 Deviating Melting Curves** and **7.6 Rare Variants.**

In case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual identification of the melting curves (Tm calling) and compare the Tm results with table 7 (**7.7. Interpretation of the Results**).

7.5.3 Melting Analysis: LightCycler® 480 Instruments

The melting-curve peaks (Fig. 5 and 6) display all genotypes.

Activate Color Compensation!
Analyzing data without 'Color Compensation' (deactivated) will generate invalid readouts of the results.

View data for Melting as follows:

LightCycler® 480 Instrument: view AAT Pi*S Melting data in channel 483-533 and AAT Pi*Z in channel 483-640. Analysis Type “Melt Curve Genotyping” mode.
LightCycler® 480 II Instrument: view AAT Pi*S Melting data in channel 465-510 (Figure 5) and AAT Pi*Z in channel 498-640 (Figure 6). Analysis Type “Melt Curve Genotyping” mode.
LightCycler® Z480 Instrument: view AAT Pi*S Melting data in channel 465-510 and AAT Pi*Z in channel 498-645. Analysis Type “Melt Curve Genotyping” mode.
LightCycler® Z480 Instrument: view HFE H63D S65C Melting data in channel 465-510 and HFE C282Y in channel 498-645. Analysis Type “Melt Curve Genotyping” mode.

Channel 465-510

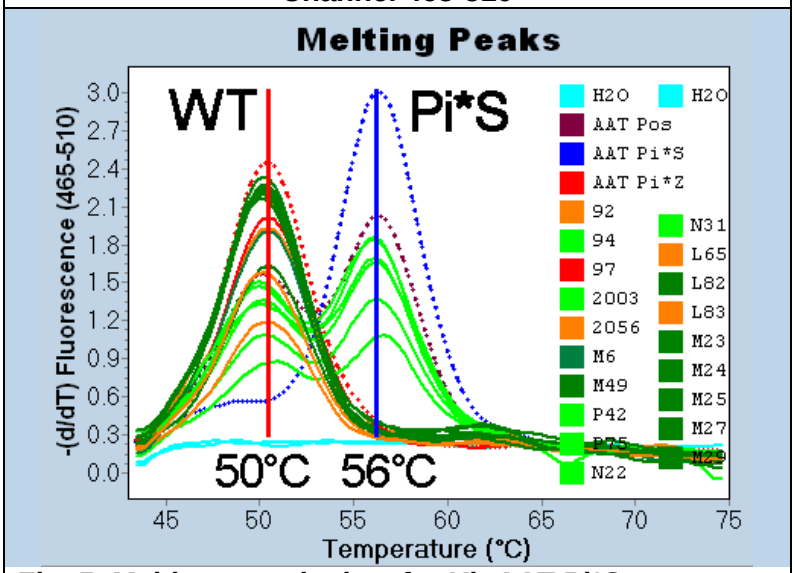


Fig. 5: Melting sample data for Kit AAT Pi*S.

NTC Negative Control
(light blue line – position 1)
no assay-specific melting peaks must be detected.
AAT Pos Positive Control
(burgundy dotted line – position 2) shows a melting peak at 50°C and a melting peak at 56°C as Pi*S heterozygous samples (E264V) (light green lines).
AAT Pi*S Genotyping Standard
(blue dotted line – position 3)
shows a melting peak at 56°C mimicking Pi*S mutant samples (264V).
Pi*Z Standard (red dotted line - position 4) shows a melting peak at 50°C as Pi*S wild type samples (E264) (dark green, red and orange lines).

Channel 498 - 640

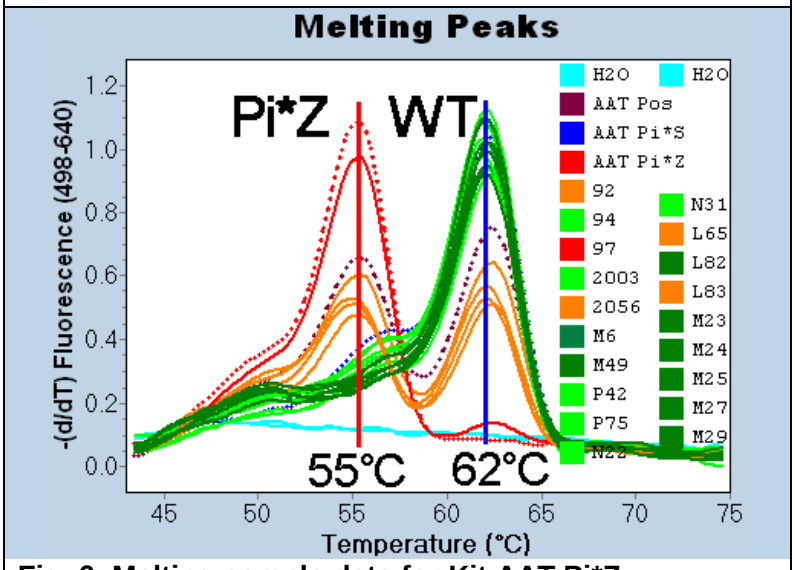


Fig. 6: Melting sample data for Kit AAT Pi*Z.

NTC Negative Control
(light blue line–position 1)
no assay-specific melting peaks must be detected.
AAT Pos Positive Control (burgundy dotted line – position 2) shows a melting peak at 55°C and a melting peak at 62°C as Pi*Z heterozygous samples (E342K) (orange lines).
AAT Pi*S Genotyping Standard
(blue dotted line – position 3) shows a melting peak at 62°C as Pi*Z wild type samples (E342) (green lines).

AAT Pi*Z Genotyping Standard (red dotted line – position 4) shows a melting peak at 55°C as Pi*Z mutant samples (342K) (red lines).

Note: The values of the melting temperatures may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.
In case of variations see instructions: **7.3.5 Deviating Melting Curves** and **7.6 Rare Variants**

In case of automatic genotype module failure, switch to manual identification of the melting curves (Tm calling) and compare the Tm results with table 7 (**7.7. Interpretation of the Results**).

7.5.4 Melting Analysis: LightCycler® Nano Instrument

The melting-curve peaks (Fig. 7 and 8) display all genotypes.

View data for Melting as follows:

Analysis

In window: **Select Analysis**	Select: Tm Calling
In window: **Setting**	Select: Use negative Derivative "Yes"
	Select: Noise Reduction Range (°C) = 1
	Select: Target: Channel 530 for **Pi*S**
	Select: Target: Channel 640 for **Pi*Z**

Melt Peaks

Manually compare the melting curve of each patient with the melting curve of Standards in both channels and use table 7 in chapter **7.7. Interpretation of the Results** for comparison.

Channel 530

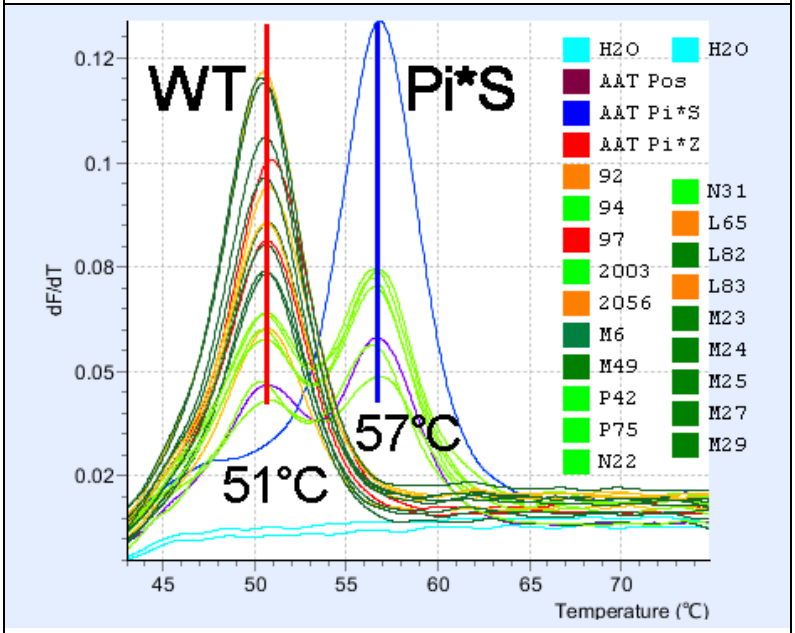


Fig. 7: Melting sample data for Kit AAT Pi*S

NTC Negative Control (light blue line – position 1) no assay-specific melting peaks must be detected.
AAT Pos Positive Control (burgundy line – position 2) shows a melting peak at 51°C and a melting peak at 57°C as Pi*S heterozygous samples (E264V) (light green lines).
AAT Pi*S Genotyping Standard (blue line – position 3) shows a melting peak at 57°C mimicking Pi*S mutant samples (264V).
Pi*Z Standard (red line - position 4) shows a melting peak at 51°C as Pi*S wild type samples (E264) (dark green, red and orange lines).

Channel 640

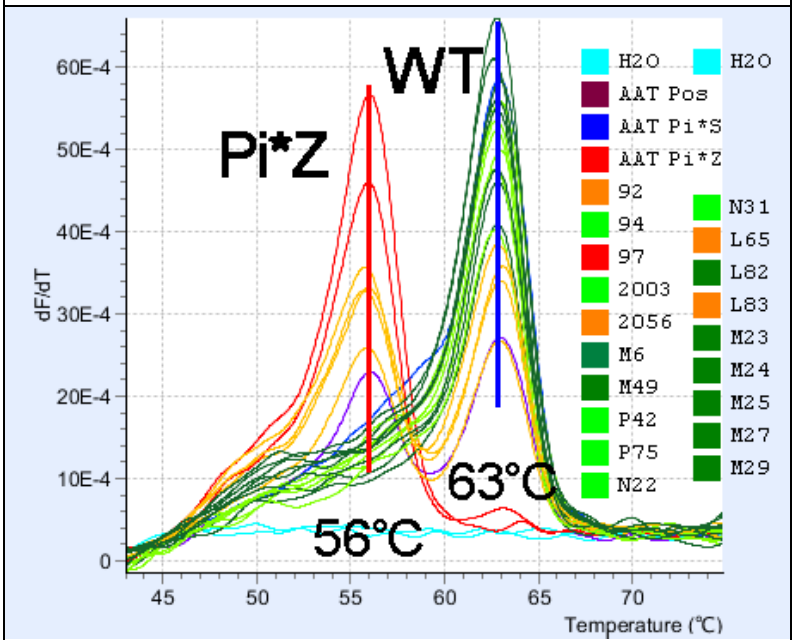


Fig. 8: Melting sample data for Kit AAT Pi*Z

NTC Negative Control (light blue line–position 1) no assay-specific melting peaks must be detected.
AAT Pos Positive Control (burgundy line – position 2) shows a melting peak at 56°C and a melting peak at 63°C as Pi*Z heterozygous samples (E342K) (orange lines).
AAT Pi*S Genotyping Standard (blue line – position 3) shows a melting peak at 63°C as Pi*Z wild type samples (E342) (dark / light green lines).
AAT Pi*Z Genotyping Standard (red line – position 4) shows a melting peak at 56°C as Pi*Z mutant samples (342K) (red lines).

Note: The values of the melting temperatures may vary ±2.5°C between different experiments. The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.

In case of variations see instructions: **7.3.5 Deviating Melting Curves** and **7.6 Rare Variants**

7.6 Melting Temperatures expected for rare variants

There are several AAT gene variations reported in dbSNP, including four SNP located near to G11940A (rs28929474) covered by the probe used in the kit. For three of them there is no allele frequency reported and these variants have to be expected to be very rare, while dbSNP reports for variant G11937A (rs143370956) a frequency of 0.1%.

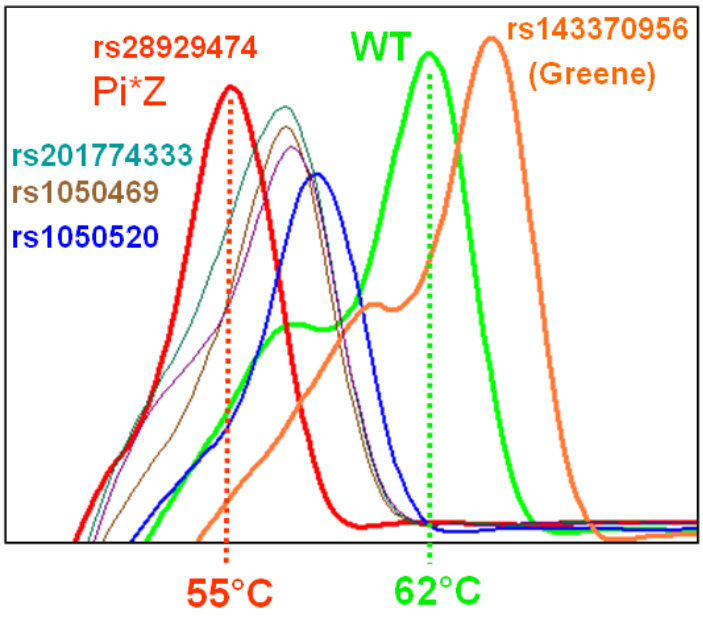
The probe used in this kit was designed to identify the variant 11937A in order to circumvent to achieve false Pi*Z results as reported by Greene et al.[6] for other published assays for AAT genotyping.

Melting temperatures obtained with synthetic controls for all known variants near Pi*Z (rs28929474) are listed in the table below :

Position	dbSNP rs number	ΔTm	Comment
11937A	rs143370956	+ 2.3°C	0.1% Greene et al.[6]
11940G	-	[62°C]	wild type
11945G	rs1050520	- 4.3°C	
11939G	rs201774333	- 4.8°C	
11939T	rs201774333	- 4.9°C	
11933G	rs1050469	- 5.0°C	
11940A	rs28929474	- 7.0°C	Pi*Z mutation

Tab. 6: Melting temperatures

In the figure below, melting peak obtained with synthetic controls are shown.



Instrument: LC 2.0

Channel 640

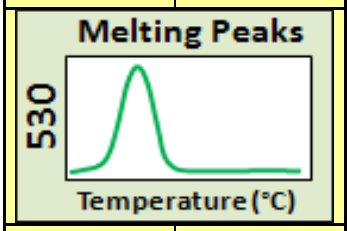
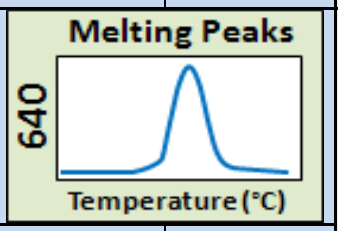
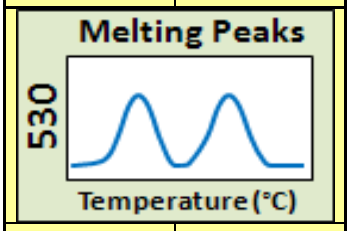
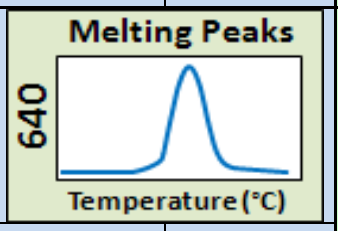
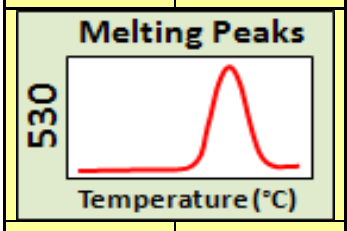
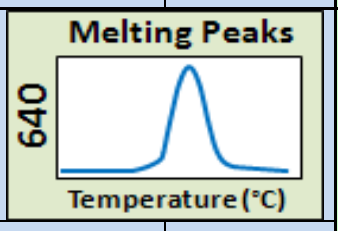
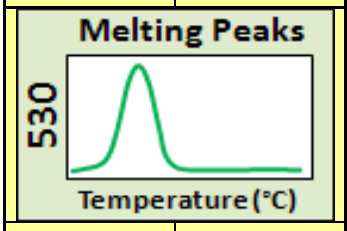
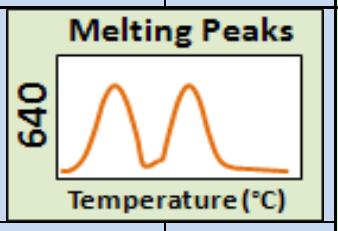
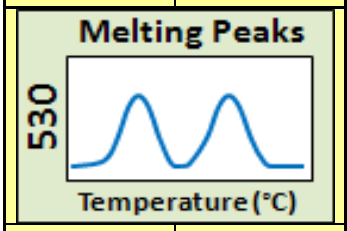
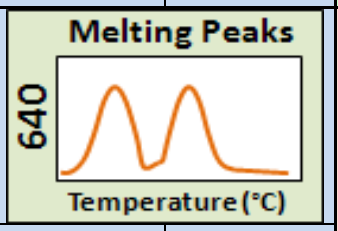
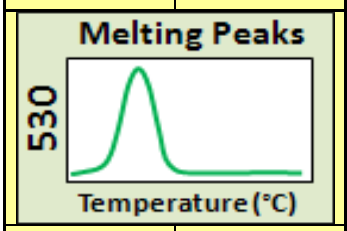
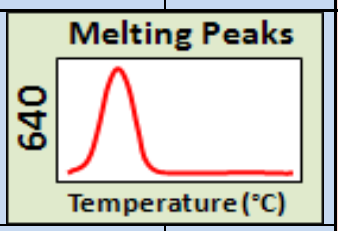
Wild type (green) and Pi*Z rs28929474 (red) can be clearly distinguished from the four intermediate melting AAT variants rs1050469 (brown line), rs1050520 (blue), and rs201774333 G or T (turquoise and violet lines), and from the higher melting variant rs143370956 (orange) as reported by Greene et al.[6]

Fig. 9: Sample melting curves obtained with synthetic oligonucleotide targets

There is currently no information about AAT deficiency related to any of these variations available in the literature.

Notes:

7.7. Interpretation of the Results

AAT Pi*S Channel 530 Melting peak(s)		AAT Pi*Z Channel 640 Melting peak (s)		AAT Genotype	AAT serum levels
WT	Pi*S	Pi*Z	WT	Amino acids	Associated Risk
				Pi MM 264 EE 342 EE (wild type)	Normal serum AAT (1.5-3.0 g/l)
49-51	-	-	62-63		
				Pi MS 264 EV 342 EE	Normal serum AAT (1.0-2.7 g/l)
49-51	55-57	-	62-63		
				Pi SS 164 VV 342 EE	Normal serum AAT (1.0-1.4 g/l) Slightly increased risk for Emphysema
-	55-57	-	62-63		
				Pi MZ 264 EE 342 EK	Normal serum AAT (0.9-2.0 g/l) Slightly increased risk for Emphysema
49-51	-	55-56	62-63		
				Pi SZ 264 EV 342 EK	Reduced serum AAT (0.5-1.2 g/l) Increased risk for Emphysema (< 50%) Cirrhosis risk
49-51	55-57	55-56	62-63		
				Pi ZZ *or* Pi ZO 264 EE 342 KK	AAT deficiency (0.2-0.5 g/l) High Emphysema risk (80-100%) Cirrhosis risk (20%)
49-51	-	55-56	-		
No 530 peak !		*No 640 peak !*		Pi 00 AAT deficiency	AAT (< 0.2 g/l) Emphysema risk

AAT gene deletion is very rare. This kit does not contain a control reactions to differentiate between PCR inhibition, missing DNA, or presence of the gene deletion. Repeat run and contact a specialist.

ΔTm 6°C	ΔTm 7°C

Note: Melting temperatures Tm may vary ±2.5°C between runs while the ΔT are rather constant.

Tab. 7: Typical analysis results

8. Troubleshooting

Instrument specific codes:	Capillary based instruments	LightCycler® 480 instruments
	LightCycler® Nano	
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All negative PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline "Saw teeth like"	Bubble in well	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in **NTC** Negative Control	Error while setting the instrument	Check the position settings of the Negative Control
	Dispensing error	Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR- grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
	PiOO allele	AAT deficiency
Melting curve outside the expected temperature range	With peaks Tm **concordant** with Positive Control: Incorrect reagent concentration	Manually assign results accordingly to Positive Control
	With peaks Tm **discordant** with Positive Control: Possible extraction inhibitor	Repeat assay diluting the DNA 1:3
	With peaks Tm **discordant** with Positive Control: Possible different mutation	Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de

9. References

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Clin Chim Acta (2011)

Classification / References

Reference	Classification
EDMA	16 01 01 90 00
CPV	33694000-1
EAN	4260159331912
Roche SAP No.	07055684001

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Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
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According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements or change in composition

Version	Event	Date
V121024	Release Version	30-04-2013
V121024C	Corrections Contents, section 6.2.2 and 6.3.1 volume	06-06-2013
V130704	MagNa Pure 96 and MagNa Pure Compact included. Instructions for automated genotyping module failure (7.5.2)	04-07-2013
V150101	Editorial changes	19-12-2014
V160626	Storage (1.1, 1.4)	26-06-2016

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