LightMix® in-vitro diagnostics kit FGB -455G/A

Cat.-No.: 40-0645-64

Detection of the G-455A DNA variation
in the FGB gene

for use with the

Roche Diagnostics LightCycler® Instruments

SimpleProbe® format

Reagents for 64 reactions

Upon arrival:

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

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

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1. Product Information

1.1. Contents: LightMix® Kit FGB

Lyophilized premixed PCR reagents:

⚠ **Store at room temperature in the dark**

	Cap color	Label	Description content	Total Reactions
1 x	**Red**	**PSR**	Parameter Specific Reagents (PSR) containing premixed and lyophilized primers and probes for 64 reactions. <0,01pg unlabeled oligonucleotides; <0,01pg SimpleProbe 519 labelled probe	64 green-blue pellet lyophilized

Standards (Control DNA)

⚠ **Store at room temperature**

	Cap color	Label	Description content	Total Reactions
1 x	**Yellow**	**HT**	Positive Heterozygous Control <0,01pg plasmids target mix (synthetic) [about 10E4 genome equivalents]	40 blue pellet lyophilized
1 x	**Yellow**	**WT**	Genotyping Standard Wildtype *<0,01pg plasmids target mix (synthetic)* *[about 10E4 genome equivalents]*	40 blue pellet lyophilized
1 x	**Yellow**	**MT**	Genotyping Standard Mutant *<0,01pg plasmids target mix (synthetic)* *[about 10E4 genome equivalents]*	40 blue pellet lyophilized

Polymerase Mix: LightCycler® FastStart DNA Master HybProbe

⚠ **Store at -20℃ upon arrival**

	Cap color	Label	Description content	Total Reactions
1 x	**Red**	**1a**	LightCycler® FastStart Enzyme	64 frozen
1 x	**White**	**1b**	LightCycler® FastStart Reaction Mix HybProbe	64 frozen
1 x	**Colorless**	**Water**	H_2O PCR grade	frozen
1 x	**Blue**	**$MgCl_2$**	$MgCl_2$, 25 mM	frozen

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 kits supplied through Roche Diagnostics or its local distributor.

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

1.2. Intended Use

This kit allows to determine the Fibrinogen beta (FGB) promoter polymorphism G/A in position -455 (FGB:c.4577G>A; rs1800790).

The FGB -455 genotype alone does not allow predictions on a disease risk.

The kit is not intended to be the only basis for therapy decision. The 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).

1.3. Specifications

The *LightMix® Kit FGB* is an *in-vitro* diagnostic test and allows the detection of the FGB G-455A single nucleotide polymorphism (SNP) as demonstrated with reference samples.

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:

Roche PCR 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(5) + 2 ctrl.	60	20
LC96	1.6 (6)	100 min	94(5) + 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 one heterozygous control and one No-Target Control (NTC) for a total of 2 control reactions.

3 The first run of the kit requires to include 4 controls to teach the genotyping module (not LC Nano, and LC96). 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 one single clinical sample analyzed in each run.

5 It requires using more than one kit.

6 Nano LightCycler® software 1.0 and LC96 software 1.6 do not contain the automatic genotyping module, therefore it is not necessary to add two 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!

Reagents and Controls

Store the lyophilized reagents (PSR and Standards) protected from light and at room temperature (18℃ / 25℃).
Do not freeze these dry reagents. Expiration date is printed on the kit label.

Polymerase mix

Store the LightCycler® FastStart DNA Master HybProbe at -15℃ to -25℃.
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

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® z 480 Analyzer	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® 96 Instrument	**Roche Diagnostics**
LightCycler® 96 Instrument	Cat.-No. 05 815 916 001
LightCycler® Software Version 1.0 or higher	Included with Instrument
LightCycler® 480 Multiwell Plate 96 white	Cat.-No. 04 729 692 001
LightCycler® 8 tube strips (white)	Cat.-No. 06 612 601 001
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:	**Roche Diagnostics**
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

The *Fibrinogen beta (FGB)* gene encodes the fibrinogen beta chain, a subunit of the fibrinogen protein. This protein is important for blood coagulation to stop excessive bleeding after injury. To form fibrinogen, the beta chain attaches to two other proteins called the fibrinogen alpha and fibrinogen gamma, Two sets of this three-protein complex combine to form functional fibrinogen.
For coagulation to occur, thrombin has to remove two fragments, the A and B fibrinopeptides from the two subunits of the functional fibrinogen protein, thus converting fibrinogen to fibrin, the main protein in blood clots. Fibrin attach to each other, forming a stable network that makes up the coagulation.
Cariers of the -455 AA allele are reported to have higher fibrinogen levels [1]. However, according to literature there is no association to disease risk based on the -455 genotype alone, while there are several studies in particular on the risk for myocardial perfusion [2], pulmonary embolism [3], or on the C-reactive protein levels during inflammation[4] made in conjunction with other gene variations such as Factor V Leiden, Factor II (prothrombin), Factor XIII, or PAI-1.
Other mutations in the *FGB* gene can cause different bleeding disorders.
Mutations in both *FGB* gene copies - most of them encoding truncated protein - lead to congenital *afibrinogenemia*, a condition that causes excessive bleeding due to the absence of fibrinogen protein in the blood.
Hypofibrinogenemia, characterized by decreased levels of fibrinogen, is caused by mutations that reduce but do not eliminate the production of the fibrinogen β chain. Individuals with hypofibrinogenemia can have bleeding problems that vary from mild to severe.
Dysfibrinogenemia is characterized by abnormally functioning fibrinogen, although the protein is present at normal levels. This condition is usually caused by mutations that change a single protein building block (amino acid) in the fibrinogen β chain. These mutations alter the function of the fibrinogen protein and, depending on the functional change, can lead to excessive bleeding or abnormal blood clotting (thrombosis).
Hypodysfibrinogenemia is a condition characterized by low levels of abnormally functioning fibrinogen protein in the blood. As in dysfibrinogenemia, this condition can result in excessive bleeding or thrombosis.

3.2. Methodology and Assay Principle

Using PCR methodology, a 198 bp fragment of the FGB promoter is amplified with specific primers. The PCR fragment is analyzed using an internally labeled SimpleProbe® oligomer binding to the region spanning the mutation site.
During the melting curve analysis the temperature is slowly increased. The probe leaves at a specific temperature (Tm) causing a fluorescence decrease. Any mismatch covered by the probe destabilizes the hybrid and lowers the Tm.
In this product the probe matches the sequence of the mutant genotype and the presence of the wild type genotype will yield a reduced Tm.
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 module (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.

3.3. Performance Characteristics

Analytical Specificity

The specificity to the target gene and the suitability of the PCR amplification employed in the present test was demonstrated by sequencing of the amplicon.

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 94 different genomic DNA samples from Caucasian origin individuals were analyzed in parallel with a previously established kit.
Study results: results for both analytical methods were in 100% concordance.
In particular, 58 samples were homozygous wild type (62%), 32 samples were heterozygous (34%), and 3 samples were homozygous mutant (3%).
Reported allele frequencies in the Caucasian population are 73%, 22% and 5%, respectively.
In one sample, identified as homozygous wild type, an additional melting peak at a Tm of approximately 47°C was visible. Sanger sequen cing confirmed the presence of the SNP -450 C/T which is registered as rs2227392; the published frequency is 2-4% in African-orign Americans and less than 1% in Causasians.
For SNP rs2227392 there is no association with a disease risk known (see 7.8.2 Rare Variants).

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 with filter 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

No Color Compensation is required for the use of this kit; reading data with 'Color Compensation' activated will not change the readout of the results.

5.2. Capillary Based LightCycler® Instruments

For details see the instrument operator’s manual.

Programming

The protocol consists of four program steps:

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 [℃]	**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 [℃/s]	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target [℃]	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Size [℃]	**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

Tab. 1. **Programming of capillary based Instruments**

Note:

While programming maintain default software values: channel = 530, max. samples = 32, seek temperature = 30℃ 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 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.

LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' instead of ‘Ramp Rate’.

5.3. Roche 480 Instruments

For details see the instrument operator's manual.

Detection Format: SimpleProbe

Note: This kit can be run in combination with LightMix® Kit HFE H63D S65C C282Y CE (cat. 40-0340-32) following the instruction for the Detection Format and Programming described in the HFE kit manual.

Reaction Volume: 10 µl

Programming

The protocol consists of four program steps:

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 [℃]	**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 ℃]	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**
Sec Target [℃]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size [℃]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay [cycles]	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 2. **Programming of 480 Instruments family**

Note:
Store the program and the default values as '**RUN Template'** which can be loaded to start every run.
Ensure to program **2 acquisitions per second** instead the default value 5; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.

5.4. LightCycler® 96 Instrument

For details see the instrument operator's manual.

Measurement

Detection Format: 470/514 FAM			**General**
Quant Factor	Melt Factor	Integration Time (S)	Volumes (µl)
10.00	1.20	Dynamic	10

Programming

The protocol consists of four program steps:

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:								
Cycles	**1**	**45**			**1**			**1**
Ramp [℃/ s]	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.20**	**1.5**
Duration [s]	**600**	**5**	**10**	**15**	**30**	**120**	**1**	**30**
Target [℃]	**95**	**95**	**60**	**72**	**95**	**43**	**75**	**40**
Mode		**Standard**	**Standard**	**Standard**				
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Readings /℃							**5**	

Tab. 3. **Programming of LightCycler® 96 Instrument**

Note:

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

5.5. LightCycler® Nano Instrument

For details see the instrument operator's manual.

Run Setting / Optical setting

Intercalating Dyes
Normal Quality

Programming

The protocol consists of four program steps:

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. 4. **Programming of LightCycler® Nano Instrument**

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

6. Experimental Protocol

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

6.1. Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin blood 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 (see 7.8.1. Typical Data for Amplification) 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

►	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 tube's 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

►	**HT Positive Control** tube is sufficient for 40 reactions.
1	Spin the **HT** tube at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the tube's bottom
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 **Positive Control** for a 10 µl PCR reaction.
► **Positive Control** must be used in each run.
Please note: Opening the vial 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 and later (LightCycler®480 instruments) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples.

►	**WT** and **MT** Genotype Standards are sufficient for 40 reactions.
	If not used, keep the Genotyping Standards lyophilized; dispose reagents when the kit is used up or after reaching the expiration date.
1	Spin the **WT** and **MT** tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the tube's bottom.
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 **WT** and **MT** 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. See chapter 6.4 for storage and stability of dilute components.
For the preparation of reaction mix for less samples, 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:	
H2O, PCR-grade (colorless cap)	343.2 µl
Mg2+ 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. 5. **Volumes of components for preparing 64 reaction mixtures**

6.3.2. Preparation of the Single LightCycler® Reaction Mix

Prepare the reaction mix by multiplying each volume (Tab. 6) 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. 6. **Volumes of components for preparing single reaction mixture**

Gently pipette up and down the reaction mix.
A high percentage of experimental failures is due to a non homogeneous reaction mix!

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 FGB PCR product and **Positive Control** to identify run specific melting temperatures. Regulatory agencies or local laboratory rules might require including also the two Genotyping Standards.

►	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**) Add **2 µl** of **HT Positive Control**.
	Optional*: Add **2 µl** of **WT** Genotyping Standard. Add **2 µl** of **MT** Genotyping Standard.
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℃ - 8℃) for 30 days.

Avoid prolonged exposure to light.

Parameter Specific Reagents (PSR)

Once diluted, store PSR refrigerated at 4℃ - 8℃ 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℃ - 8℃) for 30 days.

Positive Control

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

Genotyping Standards

The dissolved **Genotyping Standards** are stable for 30 days when stored refrigerated (4℃ - 8℃).

6.5. Loading of Controls and Genotyping Standards

Samples descried as positions 1 and 2, must be filled in each run; samples 3 and 4 are required for teaching of Genotyping Standards (only in the first run of the kit).

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.

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 deselect all others. From the pull down menu select "Sample Type" and copy the "Genotype" description.

Pos	Sample Name	Channel	Target Name	Sample Type	Genotype
1	**NTC**	530	Target 1	Negative Control	
2	**HT**	530	Target 1	Melting Standard	FGB G-455A Heterozygous
3	**WT**	530	Target 1	Melting Standard	FGB G-455 Wildtype
4	**MT**	530	Target 1	Melting Standard	FGB -455A Mutant

6.5.2. Roche 480 Instruments

In the "Sample Editor" window, in "Step1: Select Workflow" section, select "Melt Geno", filter combination 465-510. Input the description of **Positive Control** and **Genotyping Standards** as follows:

Pos	Sample Name	Melt Geno Sample Type	Melt Geno Genotype
1	**NTC**	Negative Control	
2	**HT**	Melting Standard	FGB G-455A Heterozygous
3	**WT**	Melting Standard	FGB G-455 Wildtype
4	**MT**	Melting Standard	FGB -455A Mutant

6.5.3. LightCycler® 96 Instrument

In the "Sample Editor" window input, as described below, the description of **Positive Control** and optionally **Genotyping Standards**.

Table View:

Color	Position	Sample Name	Sample Type	Dye
	A1	**NTC**	Unknown	FAM
	A2	**HT**	Unknown	FAM
	A3	**WT**	Unknown	FAM
	A4	**MT**	Unknown	FAM

Leave empty all other not described cells.

6.5.4. LightCycler® Nano Instrument

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	
	HT	
	WT	
	MT	

Target:

Color	Name	Dye	Reference
	channel 530	FAM	

Well as table:

Pos	#	Note	Sample	FAM	Type
A1	1		**NTC**	channel 530	U
A2	2		**HT**	channel 530	U
A3	3		**WT**	channel 530	U
A4	4		**MT**	channel 530	U

7. Data Analysis and Interpretation

7.1. Limits and Interferences

The present assay is specific for the FGB G-455A DNA.
No interferences are known.

7.2. Calibration

Calibration has to be performed following the procedure described in 6.5, 7.3.1, 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 **HT** 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 7.6) 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 7.3.5 and 7.6), 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 paragraph 6.5); alternatively, results must be read from the melting temperatures (see paragraph 7.7).

7.3.2. Positive Control DNA

HT Positive Control (Mandatory - position 2).
Melting-curve analysis must always show two melting peaks.
HT is mimicking an **heterozygous** clinical samples.
See **7.7 Interpretation of the Results** for expected melting temperature.

7.3.3. Genotyping Standards DNA

WT Genotyping Standard (Optional - position 3).
Melting-curve analysis must always show one single melting peak.
WT is mimicking an homozygous **wild type** clinical sample.

MT Genotyping Standard (Optional – position 4).
Melting-curve analysis must always show one single melting peak.
MT is mimicking an homozygous **mutant** clinical sample.

See **7.7 Interpretation of the Results** for expected melting temperature.

7.3.4. Samples

The result of the present assay must show one or two melting peaks.
No more than two peaks per sample are expected.

The melting peak profiles must be conformable to the acceptance criteria described in the present chapter and in **7.7 Interpretation of results**.

Before repeating a run consider common errors; check in particular the amplification profile, correct master-mix and $MgCl_2$ concentration used, and keep in mind that also inadequate storage of reagents may cause a failure of the device.

7.3.5. Abnormal Melting Curves

Unexpected melting curve might be due to an incorrect sample preparation, to a defect in the product or to a variant under the probe binding region. The whole procedure has to be repeated (sample preparation, amplification and detection). If an abnormal melting curve persists, another method must be used for identification of the sequence. Submit the PCR fragment for DNA sequencing to confirm the sequence or identify any unknown mutations.
Report deviations to service@tib-molbiol.de.
Feel free to send deviant melting samples to TIB Molbiol, GmbH, Berlin laboratories to confirm the obtained results and/or identify other mutations by DNA sequencing. Example of known variants are depicted in paragraph 7.8.2 Rare Variants.

7.4. Saving External Genotyping Standards

(Not applicable for LC1.x software versions below 4.0, LightCycler®96 and for LightCycler® Nano Instruments)

After the genotyping analysis, if samples 1 to 4 comply with the acceptance criteria (see paragraph 7.3), 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. Roche 480 Instruments

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

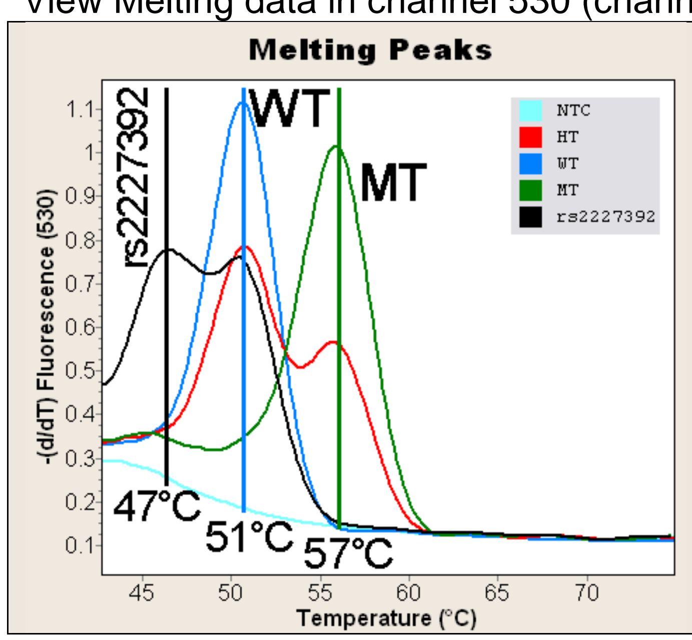
7.5. Reading the Results

Melting peaks discriminate between genotypes: heterozygous, wild type and mutant.

Use of the genotyping module present in the LightCycler® 2.0 and LightCycler® 480 software is optional; in case of automatic genotype module failure (score <0.6 or res<0.4), switch to manual identification of melting curve (Tm calling) and compare results with table in chapter **7.7. Interpretation of the Results**.

7.5.1. Melting Analysis: Capillary Based Instruments

View Melting data in channel 530 (channel F1 for *LC1.x, software version 3.5.3)*.



Channel 530

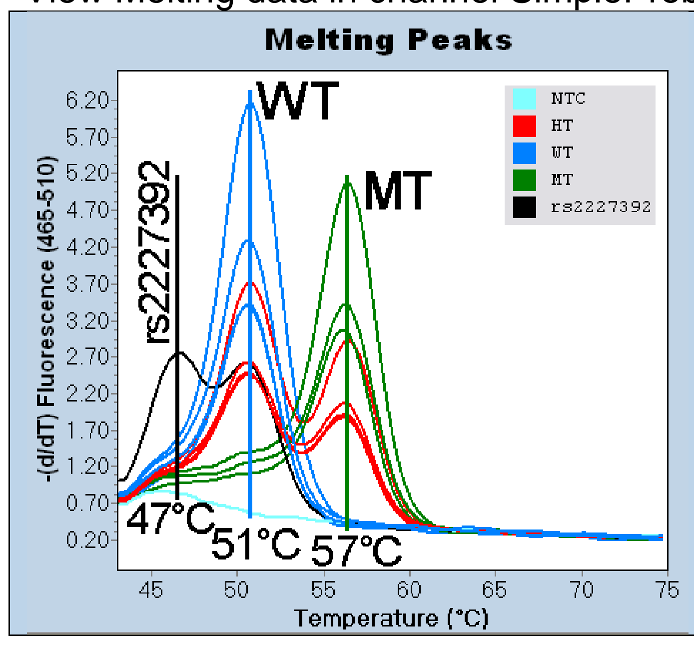
NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (red line) and heterozygous samples show melting peaks at 51°C and 57°C.
WT Standard (blue line) and homozygous wild type samples show a melting peak at 51°C.
MT Standard (green line) and homozygous mutant samples show a peak at 57°C .

A lower Tm peak at about 47°C is known for SNP rs2227392 (black line).

Fig. 1 **Capillary instruments**

7.5.2. Melting Analysis: Roche 480 Instruments

View Melting data in channel SimpleProbe.



Channel 465-510 (SimpleProbe)

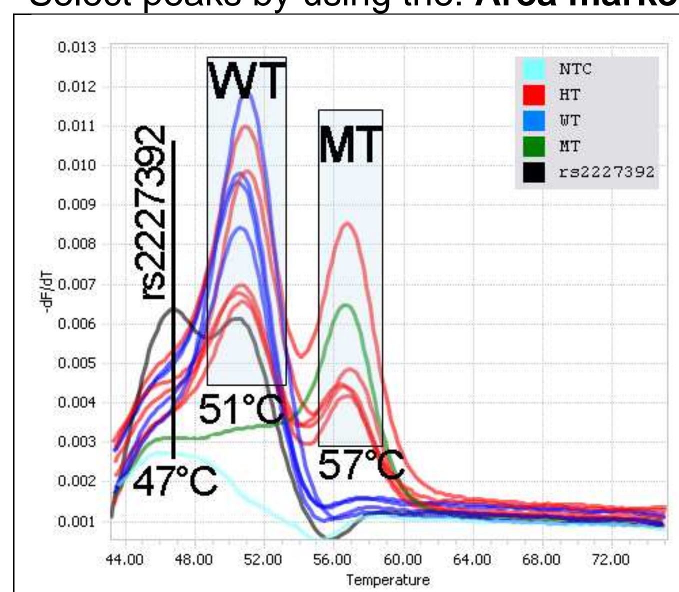
NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (red line) and heterozygous samples show melting peaks at 51°C and 57°C.
WT Standard (blue line) and homozygous wild type samples show a melting peak at 51°C.
MT Standard (green line) and homozygous mutant samples show a peak at 57°C.

A lower Tm peak at about 47°C is known for SNP rs2227392 (black line).

Fig. 2 **LightCycler® 480 Instruments**

7.5.3. Melting Analysis: LightCycler® 96 Instrument

Add Analysis: **Tm Calling**
View data in: **Melting peak**
Select peaks by using the: **Area marker tool**



Channel FAM

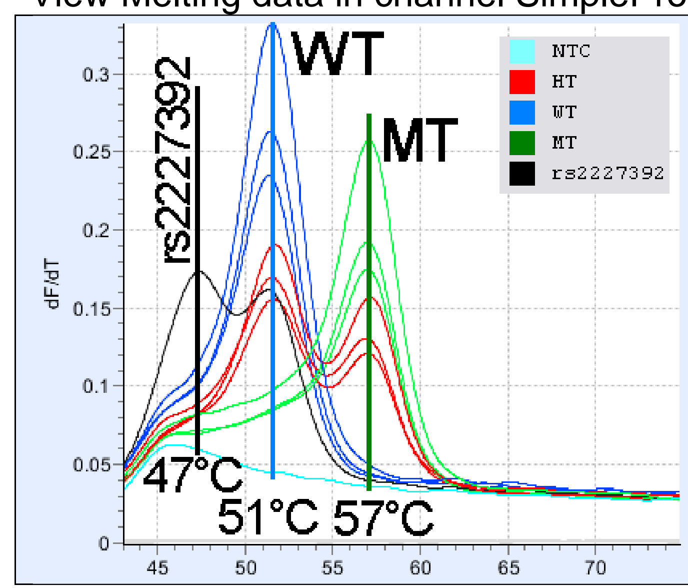
NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (red line) and heterozygous samples show melting peaks at 51℃ and 57℃.
WT Standard (blue line) and homozygous wild type samples show a melting peak at 51℃.
MT Standard (green line) and homozygous mutant samples show a peak at 57℃.

A lower Tm peak at about 47℃ is known for SNP rs2227392 (black line).

Fig. 3 **LightCycler® 96 Instrument**

7.5.4. Melting Analysis: LightCycler® Nano Instrument

View Melting data in channel SimpleProbe.



Channel 530 (FAM)

NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (red line) and heterozygous samples show melting peaks at 51℃ and 57℃.
WT Standard (blue line) and homozygous wild type samples show a melting peak at 51℃.
MT Standard (green line) and homozygous mutant samples show a peak at 57℃.

A lower Tm peak at about 47℃ is known for SNP rs2227392 (black line).

Fig. 4 **LightCycler® Nano Instruments**

7.6. Expected melting temperature

Genotype:	**mutant homozygote FGB -455A/A**	**heterozygote FGB -455G/A**	**wild type FGB -455G/G**
Number of melting peaks	**1**	**2**	**1**
Melting temperature of peaks	**56-58℃**	**50-52℃ and 56-58℃**	**50-52℃**
Temperature difference between peaks	**---**	**6℃**	**---**
Phenotype	**Higher plasma levels of Fibrinogen**	**Asymptomatic**	**Asymptomatic**

Tab. 7. **Typical analysis results**

7.7. Interpretation of the Results

FGB G-455A Channel 530 Melting peak(s)		FGB Genotypes	Metabolizers Phenotype
G-455	-455A		
Melting Peaks 530 Temperature (°C)		Mutant homozygote FGB -455A/A	Increased disease risk in conjunction with other gene variants (see 3.1 Medical Background)
-	56-58		
Melting Peaks 530 Temperature (°C)		Heterozygote FGB -455 G/A	Asymptomatic
50-52	56-58		
Melting Peaks 530 Temperature (°C)		Wild Type FGB -455 G/G	Asymptomatic
50-52	-		
ΔTm 6℃			

Tab. 8. **Typical analysis results**

Allowed variations of the melting temperatures

±0.5℃	among samples of the same genotype
±1.5℃	between genotyping standard and biological samples
±1.5℃	of ΔT among the melting peaks of heterozygous samples
±1.5℃	among melting peaks with the same genotype between runs
±5.0℃	between temperatures reported in the cartoon and values obtained by the local instruments. This variation is instrument dependent: always refer to the temperature obtained with the **HT Positive Control** included in the run.

7.8. Additional information

7.8.1. Typical Data for Amplification

The amplification curves do not contain any analytical information (see section 7.3 Quality Control – Acceptance Criteria), but, nevertheless, an example from LightCycler® 2.0 is depicted below (Fig. 5).

View data for amplification as follows:

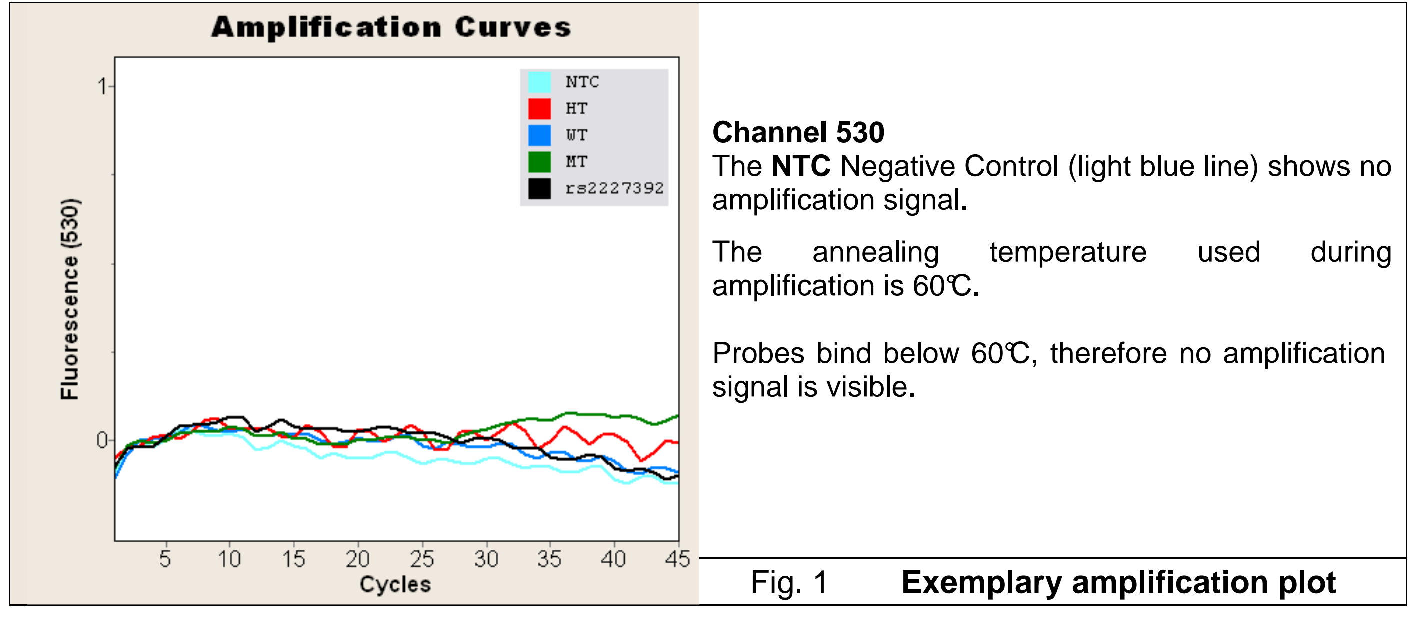
LC 2.0 Instrument (or LC1.x with software versions 4.1):
View amplification in channel 530, “Absolute Quantification” analysis mode.

LC 480 Instruments:
View amplification data in “Abs Quant/2nd Derivative Max” analysis mode.
For use in LightCycler® 480 Instrument select channel 483-533.
For use in LightCycler® 480 II Instrument select channel 465-510.
For use in in cobas z 480 Analyzer select channel 465-510.

LC 96 Instrument:
View amplification in “Abs Quant” analysis mode.

LC Nano Instrument:
View amplification in “Automatic Quantification” mode.

LC1.x, software versions 3.5 :
View amplification in fluorescence channel F1 “Quantification – Second Derivative Maximum” mode.



Channel 530
The **NTC** Negative Control (light blue line) shows no amplification signal.

The annealing temperature used during amplification is 60℃.

Probes bind below 60℃, therefore no amplification signal is visible.

Fig. 1 **Exemplary amplification plot**

7.8.2. Rare Variants

The sequences used in this device are designed to avoid to interfere with other known gene variants; new variants will usually generate a different Tm peak than WT or MT. To demonstrate the ability of the assay to discriminate the correct genotype, synthetic targets are used to mimick all the variants reported in GeneBank (Jan-2015). The absolute Tm values obtained with synthetic targets might differ from the ones resulting from biological samples, while the **relative ΔTm must remain constant**.

The present kit is not intended to identify varaints other than specified in the section **1.2 Intended Use**. Another method must be used for the identification of sequences presenting abnormal melting peaks (see **7.3.5** and **7.7**).

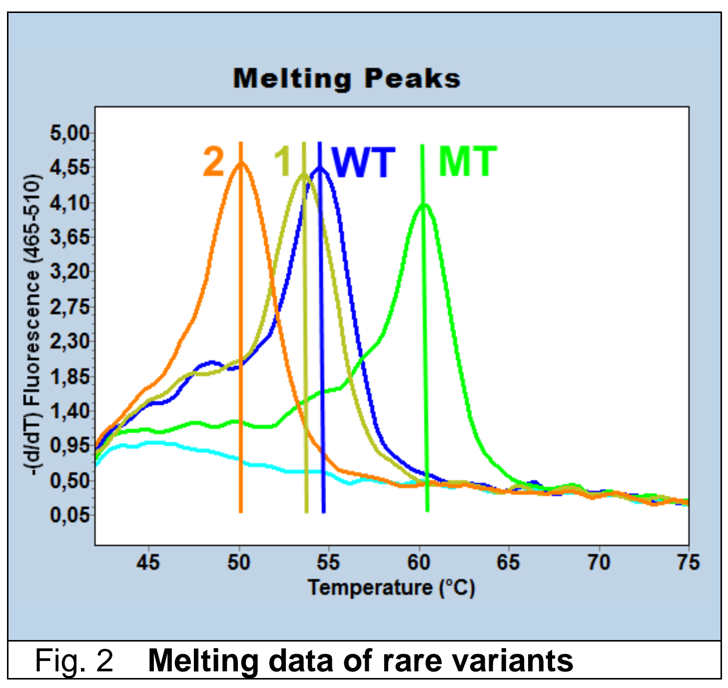


Fig. 2 **Melting data of rare variants**

#	RS	TM	HGVS	MAF
WT		54℃		
MT	rs1800790	60℃	NM_001184741.1:c.-463G>A	A=0.1414/70 8
1	rs766896403	53℃	NM_001184741.1:c.-481A>G	NA
2	rs2227392	50℃	NM_001184741.1:c.-458C>T	T=0.0016/8
3				
4				
5				
6				
7				
8				
9				
10				

MAF = Minor Allele Count (frequency of the variant); **NA**= not available

8. Troubleshooting

<table>
<tr><td rowspan="2">Instrument specific codes</td><td>Capillary based instruments</td><td>LightCycler® 480 instruments</td></tr>
<tr><td>LightCycler® Nano</td><td>LightCycler® 96 instrument</td></tr>
<tr><th>Event</th><th>Possible Reason</th><th>Solution</th></tr>
<tr><td>No sample detected</td><td>No centrifugation</td><td>Centrifuge capillary</td></tr>
<tr><td rowspan="2">All negative PCRs</td><td>Incorrect selection of detection channel</td><td>Set correct channel before analyzing</td></tr>
<tr><td>Incorrect amplification protocol</td><td>Check instrument program</td></tr>
<tr><td rowspan="3">Inconsistent baseline among various samples</td><td>Incorrect pipetting</td><td>Ensure homogeneity of mix quantity in each sample</td></tr>
<tr><td>Non homogenous reaction mix</td><td>Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel</td></tr>
<tr><td>Multiwell plate badly sealed</td><td>Ensure that multiwell plate is properly sealed</td></tr>
<tr><td rowspan="3">Baseline “Saw teeth like</td><td>Bubble in the well</td><td>Centrifuge plate before run</td></tr>
<tr><td>Bubble in the well</td><td>Centrifuge plate before run</td></tr>
<tr><td>Incorrect positioning of capillary in the carousel</td><td>Firmly press capillary in the carousel</td></tr>
<tr><td rowspan="3">No signal in the Positive Control</td><td>Error while setting the instrument</td><td>Check the position settings of the Positive Control</td></tr>
<tr><td>Incorrect PSR / MgCl2 concentration</td><td>Repeat assay</td></tr>
<tr><td>Positive Control or standard degradation</td><td>Use a new aliquot of Positive Control or standard</td></tr>
<tr><td rowspan="8">Positive signal in NTC Negative Control</td><td>Error while setting the instrument</td><td>Check the position settings of the Negative Control</td></tr>
<tr><td>Dispensing error</td><td>Comply with the work sheet when dispensing samples, Negative Controls, Positive Controls and standards</td></tr>
<tr><td>Dispensing error</td><td>Always change tips among samples</td></tr>
<tr><td>Dispensing error</td><td>Avoid spilling the contents of the sample test tube</td></tr>
<tr><td>Contamination of the PCR- grade water.</td><td>Use a new aliquot of PCR- grade water</td></tr>
<tr><td>Contamination of the reaction mix</td><td>Use new aliquots of reagents to prepare the reaction mix</td></tr>
<tr><td>Contamination of the extraction/preparation area for amplification reactions</td><td>Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use</td></tr>
<tr><td>Contamination of the extraction/preparation area for amplification reactions</td><td>Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions</td></tr>
<tr><td rowspan="2">No signal in samples</td><td>Low DNA amount</td><td>Check DNA concentration</td></tr>
<tr><td>Sample inhibition</td><td>Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat</td></tr>
<tr><td rowspan="3">Melting curve outside the expected temperature range</td><td>With peaks TM concordant with Positive Control: Incorrect reagent concentration</td><td>Manually assign results accordingly to Positive Control</td></tr>
<tr><td>With peaks TM discordant with Positive Control: Possible extraction inhibitor</td><td>Repeat assay diluting the DNA 1:3</td></tr>
<tr><td>With peaks TM discordant with Positive Control: Possible different mutation</td><td>Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de</td></tr>
</table>

9. References

(1) Papageorgiou N. et al.
Combined effects of fibrinogen genetic variability on atherosclerosis in patients with or without stable angina pectoris: Focus on the coagulation cascade and endothelial function.
Int J Cardiol. 2013 S0167-5273(13)01387-9.
(2) Satra et al.
Sequence variations in the FII, FV, F13A1, FGB and PAI-1 genes are associated with differences in myocardial perfusion.
Pharmacogenomics. 2011 Feb;12(2):195-203
(3) Klovaite et al.
Elevated Fibrinogen Levels Are Associated with Risk of Pulmonary Embolism, but Not with Deep Venous Thrombosis
American Journal of Respiratory and Critical Care Medicine, Vol. 187, No. 3 (2013), pp. 286-293.
(4) Hoope et al.,
Fibrinogen and factor XIII A-subunit genotypes interactively influence C-reactive protein levels during inflammation.
Ann Rheum Dis 2012;71:1163-1169

Classification / Reference

Reference	Classification
EDMA	16 01 04 90 00
CPV	33694000-1
EAN	4260159332629
Roche SAP No.	07163142001

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FastStart Enzyme

FastStart DNA Master HybProbe is included in the package for TIB MOLBIOL customers in Central Europe only.
When the present kit is distributed through Roche Diagnostics or their local distributors,
FastStart DNA Master HybProbe is supplied as a separate product:
Roche Diagnostics Cat.-No. 03 003 248 001 kit for 96 reactions
Roche Diagnostics Cat.-No. 12 239 272 001 kit for 480 reactions

Material Safety Data

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 and changes in composition

Version	Event	Date
V130704	Release Version	09-09-2013
V131211	EDMA reference corrected, Editorial changes	11-12-2013
V160101	Section “7.8 Additional Information” added. HGVS codes included (7.8.1).	01-01-2016

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