LightMix® in-vitro diagnostics kit Factor II G20210A

Cat.-No.: 40-0593-64
Roche SAP No: 06896502001

Detection of the G20210A DNA variation
in the Factor II (prothrombin) gene

for use with the

Roche Diagnostics LightCycler® Instruments

SimpleProbe® format

Reagents for 64 reactions

Upon arrival:

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

CE IVD

 Manual Version V240605

Table of contents

1. Product Information

1.1. Contents: LightMix® Kit Factor II G20210A

Dried premixed PCR reagents:				
	Store at 4 °C to 25 °C protected from light			
	Cap color	**Label**	**Description content**	**Total Reactions**
1 x	**Red**	**PSR**	Parameter Specific Reagents (PSR) containing premixed and dried primers and probes for 64 reactions. ≤ 71 % Synthetic oligonucleotides ≥ 29 % Buffer	64 green-blue pellet dried

Standards (Control DNA)				
	Store at 4 °C to 25 °C protected from light			
	Cap color	**Label**	**Description content**	**Total Reactions**
1 x	**Yellow**	**HT**	Positive Heterozygous Control about 4E5 genome equivalents	40 blue pellet dried
1 x	**Yellow**	**WT**	Genotyping Standard Wildtype about 4E5 genome equivalents	40 blue pellet dried
1 x	**Yellow**	**MT**	Genotyping Standard Mutant about 4E5 genome equivalents	40 blue pellet dried

1.2. Intended Use

This diagnostic PCR kit allows the detection of the Factor II (F2, prothrombin, OMIM 176930) G20210A mutation (NCBI dbSNP: rs1799963) from genomic human DNA extracted from peripheral blood.

This F2 mutation belongs, like Activated Protein C (APC) resistance (Factor V Leiden), or deficiency of Antithrombin III, Protein C or Protein S, to the group of heredity risk factors for thrombosis.

This product is indicated to help the clinician to analyze the genetic back-ground of patients showing :

- venous thrombosis
- pulmonary embolism
- premature ischemic stroke
- premature myocardial infarction in women
- family history for the factor II mutation
- family history of stroke, pulmonary embolism, deep vein thrombosis in first degree relatives under 50.

As the risks for thrombosis increases for those individuals bearing Factor II and Factor V mutations, patients should be analyzed also for the Factor V (Leiden) polymorphism G1691A, for example using the LightMix® Kit 40-0594 (Roche SAP No.: 06896529001).

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 Factor II G20210A* is an *in-vitro* diagnostic test and allows the detection of the Factor II G20210A 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 reaction volume.
Each run must contain at least one standard and one negative control.
For LightCycler® PRO, all three standards and one negative control have to be used for each run.

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
z 480 (open channel)	1.5	100 min	94(5) + 2 ctrl.	60	20
LC96	1.6	100 min	94(5) + 2 ctrl.	60	20
LC PRO	1.X.X	70 min	92(6) + 4 ctrl.	60	12

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 use of the kit requires to run 4 controls to teach the genotyping module (not 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 Each run must include one heterozygous control (HT), both Genotypinge Standards (WT and MT) and one No-Target Control (NTC).

1.4. Storage and Stability

Reagents and Controls

Store the dried reagents (PSR and Standards) protected from light and at room temperature or refrigerated (4 °C to 25 °C).
Do not freeze dried reagents.
The expiration date is printed on the kit label.

Shipping

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

2. Additional Devices and Reagents

2.1. Required

LightCycler® 2.0 Instruments	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Discontinued
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Discontinued
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 Instruments	**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® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 898 915 001
LightCycler® Capillaries (20 µl)	Discontinued
Or	
LightCycler® PRO Instruments	**Roche Diagnostics**
LightCycler® PRO Instrument	Cat.-No. 09 541 713 001
LightCycler® Software Version 1.X.X	Included with Instrument
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

Optional:

2.2. Instruments:

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	Discontinued
MagNA Pure LC DNA Isolation Kit I	Cat.-No. 03 003 990 001
MagNA Pure Compact Instrument	Discontinued
MagNA Pure Compact Nucleic Acid Isolation Kit I	Cat.-No. 03 730 964 001
MagNA Pure 96 Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001
MagNA Pure 24 Instrument	Cat.-No. 07 290 519 001
MagNA Pure 24 Total NA Isolation Kit	Cat.-No. 07 658 036 001

2.4. Reagents

	Roche Diagnostics
LightCycler FastStart DNA Master HybProbe	Cat.-No. 12 239 272 001

3. Background Information

3.1. Medical Background

The thrombin gene encodes the Coagulation Factor II (F2 or Prothrombin, OMIM: 176930), a Vitamin K-dependent glycoprotein synthesized as inactive proenzyme by the liver.

The G20210A transition, located in the 3-prime untranslated region of the gene (c.*97G>A), is associated with elevated plasma prothrombin levels and a 3-fold increased risk of venous thrombosis (Degen et al. 1987)[1].

Both heterozygous and homozygous carriers are affected.

The allele frequency in Caucasians is in the range of 1-3 %.

History

The appearance of the prothrombin G20210A mutation in Caucasians has been assigned about 23,720 years ago by linkage disequilibrium analysis. It must have appeared just after the divergence of Africans from non-Africans and of Caucasian from Mongolian subpopulations.
A similar analysis performed for the Factor V mutation yielded an estimated age of 21,340 years. The occurrence of the two mutations at the end of the last glaciation and their presently wide distribution in whites suggested selective evolutionary advantages (diminished blood loss). The selection disadvantages from thrombosis and thrombotic conditions found in the modern population were unlikely to play an important role as until recent centuries humans did not live long enough to manifest a meaningful incidence of thrombosis (Zivelin et al. 2006)[2].

Thrombosis

In a case-control study, the 20210 A allele was identified as a factor with an almost 3-fold increased risk of venous thrombosis. The risk of thrombosis increased for all ages and both sexes. An association was found between the presence of the 20210 A allele and elevated prothrombin levels. Elevated prothrombin itself also was found to be a risk factor for venous thrombosis (Poort et al. 1996)[3].

Myocardial infarction

A 4-fold increased risk of myocardial infarction in women was associated to the mutation (Rosendaal et al. 1997)[4]; a 1.5-fold increased risk was found among men (Doggen et al., 1998)[5].

3.2. Methodology and Assay Principle

Using PCR methodology, a 106 bp fragment of the Factor II gene 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 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 wild type genotype and the presence of a mutation variant will result in 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 the genotype results can be achieved by the automated genotyping module (instrument-dependent: software module 'Melt Curve Genotyping') or by the kit specific LightCycler® Analysis Package (LCAP) for LightCycler® PRO Instrument.

Automated genotyping results must be reviewed manually for deviating curves and intermediate melting point temperatures. In case that automated typing fails to report consistent genotype results, the genotype must be deduced 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 were 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 142 different genomic DNA samples from individuals of Caucasian origin were analyzed in parallel by sequencing and with the present kit. The study compared results obtained with the kit with ABI 3730xl DNA sequencing data obtained by LGC Genomics GmbH, Berlin.

Study results: Results for both analytical methods were in 99.3 % concordance.

In particular, 136 samples were homozygous wild type, 4 heterozygous and 1 homozygous mutant.
1 sample, identified by the automatic genotyping software as heterozygous G/A, has been detected by sequencing as homozygous wild type for the analyzed mutation and heterozygous for the close mutation C20209T rs 72550707 (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 workflow 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.

Always check for the latest version of the LCAP. Please visit navifyportal.roche.com to download.

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 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 °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

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

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 Factor II 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.

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

5.3. LightCycler® 480 Instruments

For details see the 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 °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	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**
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 family**

Note:

Store the program and the default values as '**RUN Template'** which can be loaded to start every Factor II LightCycler® 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 Instruments

For details see the 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 °C/ 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 °C	**95**	**95**	**60**	**72**	**95**	**43**	**75**	**40**
Mode		**Standard**	**Standard**	**Standard**				
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Readings /°C							**5**	

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

Note:

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

5.5. LightCycler® PRO Instruments

Use the software version 1.X.X. See the LightCycler® PRO System User Assistance for details.

For a matching LightCycler® Analysis Package (LCAP) file for LightMix® Kit Factor II, please visit navifyportal.roche.com to download. Please check for the latest version of the LCAP.

The kit-specific run profile is part of the LCAP and equivalent to the run conditions shown above.

LightCycler® Analysis Package: 1003_FactorII_96

Save the LCAP file in the assay folder of the SFTP or USB device.

Import and install the downloaded LCAP onto the LightCycler® PRO Instrument and activate it.

Create or import a plate setup in the Plates tab.

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 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 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 7.5.. Reading 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 HypProbe

For details see the LightCycler® FastStart DNA Master HybProbe methode sheet.

1	Keep LightCycler® FastStart Enzyme **1a** cold.
2	Thaw the LightCycler® FastStart Reaction Mix **1b** by warming up the tube at 30 °C - 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 2 °C – 8 °C.

6.2.2. Preparation of Parameter-Specific Reagents

►	The provided **PSR** 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	Add **66 µl** of PCR-grade **Water** to the **PSR** tube.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **1 µl** of dissolved **PSR** 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) and software 1.X.X (LightCycler® PRO) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples.

►	**WT** and **MT** Genotyping Standards are sufficient for 40 reactions.
	If not used, keep the Genotyping Standards dried; 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. For LightCycler® PRO both **Genotyping Standards** have to be used in **each** run.
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 reactions

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 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
$MgCl_2$ 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 Reaction Mix by single reactions

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**. For LightCycler® PRO both **Genotyping Standards, Positive Control** and **Negative Control** are mandatory for **each** run.

Prepare the reaction mix in a cooled vial:

Components	**Single reaction**
H_2O, PCR-grade (colorless cap)	5.2 µl
$MgCl_2$ 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 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 Factor II PCR product and **Positive Control** to identify run specific melting temperatures. Regulatory agencies, instrumental requirements or local laboratory rules might require including both 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**. For LightCycler® PRO both **Genotyping Standards (WT, MT)** have to be used.
	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 Dissolved Components

Reaction Mix

The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (2°C - 8°C) for up to 30 days.
Avoid prolonged exposure to light.

Parameter Specific Reagents (PSR)

The dissolved PSR is stable for up to 30 days when stored refrigerated (2 °C – 8 °C). Avoid prolonged exposure to light.

LightCycler® FastStart DNA Master HybProbe

The combined FastStart DNA Master HybProbe master mix (1a+1b) can be stored refrigerated (2 °C – 8 °C) for up to 30 days.

Positive Control

The dissolved Positive Control is stable for up to 30 days when stored refrigerated (2 °C – 8 °C).

Genotyping Standards

The dissolved **Genotyping Standards** are stable for up to 30 days when stored refrigerated (2 °C – 8 °C).

6.5. Loading of Controls and Genotyping Standards

Samples described 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. For LightCycler® PRO both **Genotyping Standards** have to be used in **each** run

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, but is mandatory for LightCycler® PRO.

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	Factor II G20210A Heterozygous
3	**WT**	530	Target 1	Melting Standard	Factor II G20210 Wildtype
4	**MT**	530	Target 1	Melting Standard	Factor II 20210A Mutant

6.5.2. LightCycler® 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	Factor II G20210A Heterozygous
3	**WT**	Melting Standard	Factor II G20210 Wildtype
4	**MT**	Melting Standard	Factor II 20210A Mutant

6.5.3. LightCycler® 96 Instruments

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

6.5.4. LightCycler® PRO Instruments

In the plate setup the matching LCAP has to be selected. Melting Standards and NTC have to be assigned to the associated well position.

Pos	Sample ID	Sample Role	Genotype
1	**1003_FactorII_NTC**	No-template Control	
2	**Factor II_HT**	Melting Standard	Factor II G20210A Heterozygous
3	**Factor II_WT**	Melting Standard	Factor II G20210 Wildtype
4	**Factor II_MT**	Melting Standard	Factor II 20210A Mutant

7. Data Analysis and Interpretation

7.1. Limits and Interferences

The present assay is specific for the Factor II G20210A 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.
For LightCycler® PRO it is essential that in addition to **NTC** and **HT** Positive Control both Genotyping Standards **WT** and **MT** are included in each run
Note: The test is performed at an annealing temperature of 60 ºC 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) may 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.

In case a peak is detected at an unspecific temperature (see paragraph 7.3.5), the software might incorrectly identify it as positive, whereby automatic genotyping is impossible.

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). Not applicable with LightCycler® PRO Instruments.

7.3.2. Positive Control DNA

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

7.3.3. Genotyping Standards DNA

Genotyping Standards are **mandatory** in **each** run performed on **LightCycler® PRO**.
WT Genotyping Standard (Optional - position 3).
Melting-curve analysis must always show one single melting peak.
WT is mimicking homozygous **wild type** clinical sample.

MT Genotyping Standard (Optional – position 4).
Melting-curve analysis must always show one single melting peak
MT is mimicking 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.
For the purpose of product improvement and post-market surveillance, please send deviant melting samples to TIB Molbiol GmbH, Berlin laboratories. Contact service@tib-molbiol.de before sending.
Examples 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® PRO 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. LightCycler® 480 Instruments

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

7.4.3. LightCycler® PRO Instruments

Genotyping Standards have to be used in **each** run.

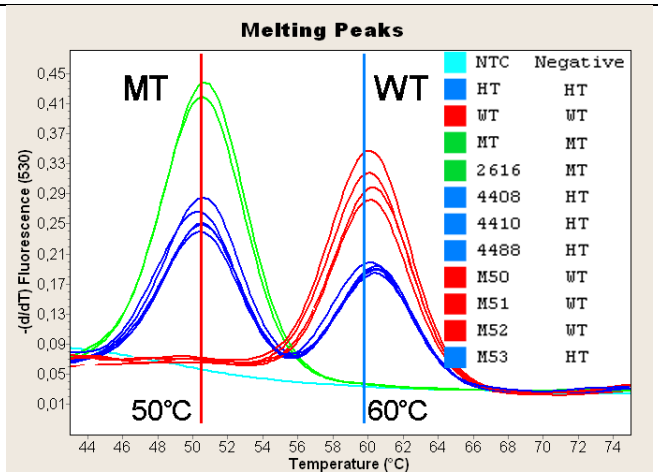
7.5. Reading the Results

Melting peaks discriminate between genotypes: heterozygous, wild type and mutant. The amplification curves do not contain any analytical information (see section 7.3 Quality Control – Acceptance Criteria).

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)*.



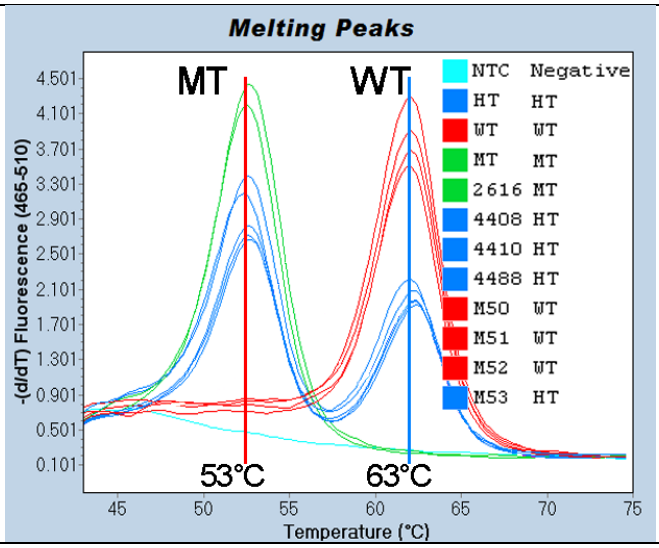
Channel 530

NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (blue line) and heterozygous samples (4408, 4410, 4488, M53) show melting peaks at 50 °C and 60 °C.
WT Standard (red line) and homozygous wild type samples (M50, M51, M52) show a melting peak at 60 °C.
MT Standard (green line) and homozygous mutant sample (2616) show a melting peak at 50 °C.

Fig. 1 **Capillary Instruments**

7.5.2. Melting Analysis: LightCycler® 480 Instruments

View Melting data in channel SimpleProbe.



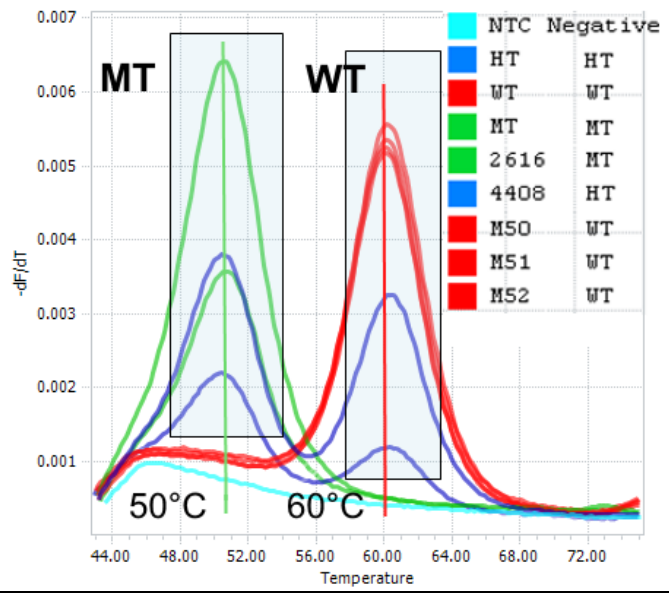
Channel 465-510 (SimpleProbe)

NTC Negative Control
(light blue line) no assay-specific melting peaks must be detected.
HT Positive Control (blue line) shows melting peaks at 53 °C and 63 °C as heterozygous samples (4408, 4410, 4488, M53).
WT Genotyping Standard (red line) shows a melting peak at 63 °C as wildtype samples
(M50, M51, M52).
MT Genotyping Standard (green line) shows a melting peak at 53 °C as mutant sample (2616).

Fig. 2 **LightCycler® 480 Instruments**

7.5.3. Melting Analysis: LightCycler® 96 Instruments

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 (blue line) shows melting peaks at 50 °C and 60 °C as heterozygous sample (4408).
WT Genotyping Standard (red line) shows a melting peak at 60 °C as wildtype samples (M50, M51 and M52).
MT Genotyping Standard (green line) shows a melting peak at 50 °C as mutant sample (2616).

Fig. 3 **LightCycler® 96 Instruments**

7.5.4. Genotyping Analysis: LightCycler® PRO Instruments

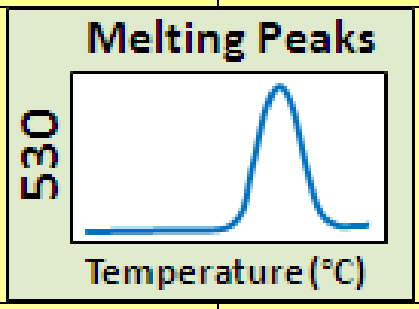
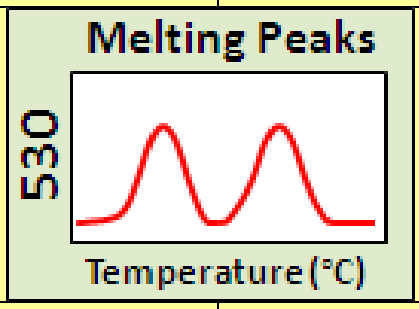
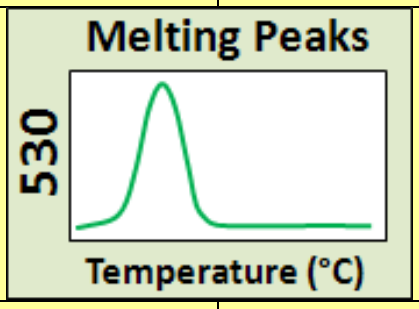
Perform data analysis as described in the LightCycler® PRO System User Assistance.
Review and approve the results in the Target Results tab. It is necessary to approve the results of melting standards (**WT**, **HT**, **MT**) and **NTC** before approving the results of unknown samples. It is possible to overwrite results, overwritten results will be flagged. In the Sample Results tab approved results can be released.

7.6. Expected Melting Temperature

Genotype:	mutant homozygote Factor II 20210A/A	heterozygote Factor II 20210G/A	wild type Factor II 20210G/G
Number of melting peaks	**1**	**2**	**1**
Melting temperature of peaks	**50 – 53 °C**	**50 – 53 °C and 60 – 63 °C**	**60 – 63 °C**
Temperature difference between peaks	**---**	**10 °C**	**---**
Phenotype	**Elevated plasma prothrombin levels**	**Elevated plasma prothrombin levels**	**Asymptomatic**

Tab. 6. **Typical analysis results performed on LightCycler® 480 Instruments**

7.7. Interpretation of the Results

Factor II G20210A Channel 530 Melting peak(s)		Factor II Genotypes	Metabolizers Phenotype
20210A	G20210		
		**Wild Type** **Factor II 20210G/G**	**Asymptomatic**
-	60-63		
		**Heterozygote** **Factor II 20210G/A**	**Elevated plasma prothrombin levels**
50-53	60-63		
		**Mutant homozygote** **Factor II 20210A/A**	**Elevated plasma prothrombin levels**
50-53	-		
ΔTm 10°C			

Tab. 7. **Typical analysis results**

Allowed variations of the melting temperatures

±0.5 °C	among samples of the same genotype
±1.5 °C	between Genotyping Standard and biological samples
±1.5 °C	of ΔT among the melting peaks of heterozygous samples
±1.5 °C	among melting peaks with the same genotype between runs
±5.0 °C	between temperatures reported here 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).

7.8.2. Rare Variants

The sequences used in this device do not 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 (Nov-2023). 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 variants other than specified in 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**).

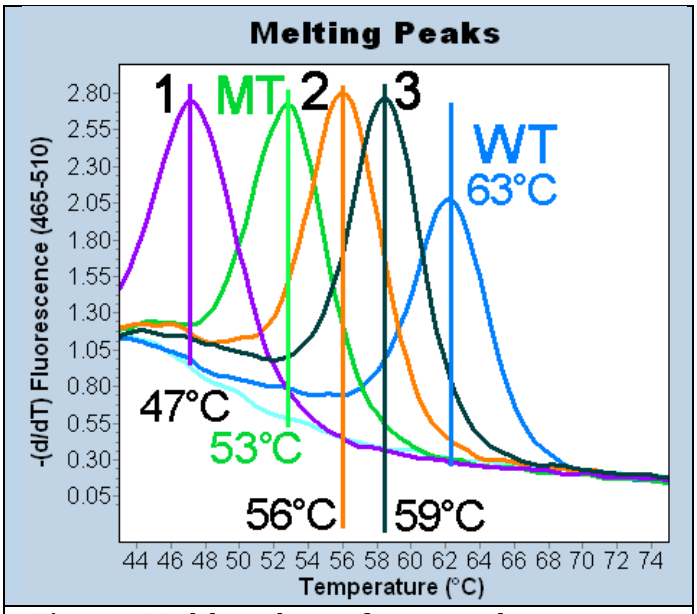


Fig. 4 **Melting data of rare variants**

#	RS	Tm	HGVS	MAF
WT		**63°C**		
MT	**rs1799963**	**53°C**	NM_000506.4:c.*97G>A	A=0.00995 (1250/125568)
1	**rs72550707 + rs1799963**	**47°C**	NM_000506.4:c.*96-97CG>TA	1 report
2	**rs72550707**	**56°C**	NM_000506.4:c.*96C>T	T=0.00102 (128/125568)
3	**rs112016113**	**59°C**	NM_000506.4:c.*100C>A	T=0.00001 (1/125568)

MAF =Minor Allele Count; **NA**= not available

Experimentally tested variants with rs code (dbSNP), Human Genome Variation Society (HGVS) nomenclature, and allele frequencies (based on TOPMed data). Temperatures (Tm) collected using synthetic targets. Tm values must not be used for prediction of genotypes. Use information in section **7.5** to **7.7**.

8. Troubleshooting

Instrument specific codes	Capillary based instruments	LightCycler® 96,480 and PRO instruments
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 the 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
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

1) Degen SJ, Davie EW.
Nucleotide sequence of the gene for human prothrombin.
Biochemistry. 1987 Sep 22;26(19):6165-77.
2) Zivelin, A., Mor-Cohen, R., Kovalsky, V., Kornbrot, N., Conard, J., Peyvandi, F., Kyrle, P. A., Bertina, R., Peyvandi, F., Emmerich, J., Seligsohn, U.
Prothrombin 20210G-A is an ancestral prothrombotic mutation that occurred in whites approximately 24,000 years ago.
Blood 107: 4666-4668, 2006
3) Poort SR, Rosendaal FR, Reitsma PH, Bertina RM.
A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis.
Blood. 1996 Nov 15;88(10):3698-703.
4) Rosendaal FR, Siscovick DS, Schwartz SM, Psaty BM, Raghunathan TE, Vos HL.
A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women.
Blood. 1997 Sep 1;90(5):1747-50.
5) Doggen CJ, Cats VM, Bertina RM, Rosendaal FR.
Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A.
Circulation. 1998 Mar 24;97(11):1037-41.

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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
V170303	Misleading wording corrected (7.3.1)	12-10-2017
V190123	Disclaimer included (7.8.2)	07-02-2019
V240329	Addition of the LightCycler® PRO and MagNa Pure24. LightCycler® Nano and FastStart DNA Master HybProbe removed. Editorial changes.	26-03-2024
V240605	Correction of version ID	05-06-2024

Report device observations, deviations and problems including lot number(s) and a brief error description to your local Roche representative.

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