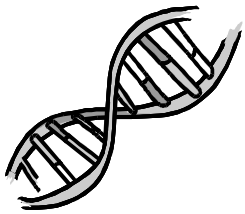
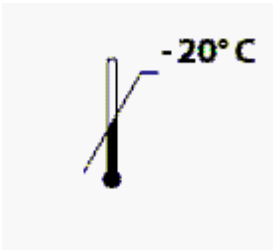
Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
fax 02-92157285
info@listarfish.it
www.listarfish.it



MutaPLATE® Factor II (TM)

(TAQ-Man) real time PCR Kit

PCR test for analysis of the G20210A mutation within the 3' UTR region of the prothrombin gene (human coagulation factor II) in open real time PCR systems (z. B. RotorGene, SmartCycler, Light Cycler, ABI, Amplifa, Stratagene) by Taq-Man technology.



IVD

in vitro Diagnostic only

CE

REF KF1901632 Σ 32

REF KF1901696 Σ 96

Table of Content

1 Intended Use

MutaPLATE ® Factor II (TM) *real time* PCR kit is a molecular biological test for the detection of the G20210A mutation within the 3' UTR region of the prothrombin gene (human coagulation factor II) in open real time PCR systems (e. g. RotorGene, SmartCycler, Light Cycler, ABI, Stratagene, Amplifa). This mutation is related to a big amount of prothrombin in plasma.

2 Introduction

Prothrombin (Factor II) is a protein in the blood required for the blood to clot. Blood clots are composed of a combination of blood platelets and a meshwork of the blood clotting protein fibrin. Prothrombin as blood clotting protein is needed to form fibrin. Persons with too little prothrombin have a bleeding tendency. If an individual has too much prothrombin, blood clots may form when they shouldn't.

Variation	RsNumber	Effect		
		Wildtype	Heterozygous	Homozygous
G20210A	rs1799963	Normal levels of prothrombin.	Intermediate increase in circulating prothrombin levels associated with higher thrombin formation. Prone to venous thrombosis.	Strong increase in circulating prothrombin levels associated with higher thrombin formation. Prone to venous thrombosis.

[1] V. D. Stefano, I. Martinelli, P. M. Mannucci, K. Paciaroni, P. Chiusolo, I. Casorelli, E. Rossi und G. Leone, „The risk of recurrent deep venous thrombosis among heterozygous carriers of both factor V Leiden and the G20210A prothrombin mutation.," *N Engl J Med,* Bd. 341, Nr. 11, pp. 801-806, 1999.

[2] A. Gerhardt, R. E. Scharf, M. W. Beckmann, S. Struve, H. G. Bender, M. Pillny, W. Sandmann und R. B. Zotz, „Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium.," *N Engl J Med,* Bd. 342, Nr. 6, pp. 374-380, 2000.

[3] R. F. Franco, M. D. Trip, H. t. Cate, A. v. den, M. H. Prins, J. J. Kastelein und P. H. Reitsma, „The 20210 G-->A mutation in the 3'-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease.," *Br J Haematol,* Bd. 104, Nr. 1, pp. 50-54, 1999.

3 Principle of the Test

MutaPLATE® Factor II (TAQ-Man) *real time* PCR Kit contains specific primers and additional material for the detection of the G20210A polymorphism of the prothrombin gene. The variable area of the prothrombin gene is amplified by PCR using **genomic DNA-template**.

The standard PCR contains additionally **two sequence specific oligonucleotides** marked with fluorescence dye (TaqMan probes). Both probes bind at the amplificated target-DNA which includes the single nucleotide polymorphism (SNP). Due to this, a fluorescence signal is generated and detected by the **optical unit** of the used *real time*

PCR instrument. The TaqMan probe for the G-allele (wildtype) is marked with **FAM (510 nm, green)** and the TaqMan probe for the A-allele (mutation) is marked with **YAK (555 nm, yellow)**.

The following three discriminations are possible:

1. Homozygous **G/G**:
   Increase of the fluorescent signal from the **FAM** labeled TaqMan probe, no increase of the fluorescent signal from the YAK labeled TaqMan probe.
2. Heterozygous **G/A**:
   Increase of the fluorescent signal from the **FAM** labeled TaqMan probe and increase of the fluorescent signal from the **YAK** labeled TaqMan probe.
3. Homozygous **A/A**:
   No increase of the fluorescent signal from the **FAM** labeled TaqMan probe, increase of the fluorescent signal from the **YAK** labeled TaqMan probe.

4 Kit Content

Each kit contains enough reagents to perform **32** respectively **96** tests. Each kit also contains a package insert.

Reference	Type of reagent	Volume (32x)	Volume (96x)
Blue	Enzymemix	435 µl	3 x 435 µl
Yellow	Detectionmix **G - Allele**	175 µl	3 x 175 µl
White	Detectionmix **A - Allele**	175 µl	3 x 175 µl
Red	Positive Control	15 µl	3 x 15 µl
Green	Negative Control	50 µl	3 x 50 µl

5 Required Materials

Provided:

- Reagents for real-time PCR
- Package insert

Not provided:

- *real time* PCR capillary system (e. g. RotorGene)
- PCR reaction tubes
- Cryo container for PCR reaction tubes
- DNA extraktion kit for isolation of genomic DNA (ca. 10 ng/µl), e.g. **MutaCLEAN® DNA Blood, KG1033,**
- Pipetts (0,5 – 200 µl) with sterile filter Tipps for micro pipets
- sterile microtubes
- gloves (powder free)

6 Storage and Handling

- All reagents should be **stored at <-20°C till immediate use**. Spin down kit components in their vials before long-term storage.
- **Avoid several freeze / thaw** cycles for the reagents (if necessary prepare suited aliquots and freeze again **immediately**).
- During preparation of PCR perform all working steps in a cryo-container (e.g. Light Cycler® Cooling block) or **cool all reagents** in suited manner.
- Primer-/ Probe-Mix should be **stored in the dark** (**light protection**).
- All reagents can be used until the expiration date (printed on the labels).

7 Warnings and Precautions

- For *in vitro* diagnostic use only.
- This assay needs to be carried out by especially in molecular biology skilled personnel: This assay needs to be run according to GLP (Good Laboratory Practice).
- Clinical samples should be regarded as potentially infectious materials.
- Mix all reagents carefully before use, but do not vortex.
- Do not use the kit after its expiration date.

8 Test Procedure

Before start, **decontaminate** all working areas and used instruments. Thaw kit components **gently at 8°C** and handle detection mixes in the dark. Prepare the necessary amount of PCR reaction tubes in a pre-cooled cooling block and consider additional 2 tubes for controls. Keep DNA samples ready and mix well before use.

Enzyme mix (*ready to use*)

This ready to use enzyme mix is stable for about 3 month at -20°C; after freezing, this solution can be thawed twice at 8°C provided that it was not stored longer than one hour (cooled) during the working steps.

Master mix preparation

Following table shows the composition for **one reaction** (final volume: 25 µl). For analysis of several samples in parallel, a **master mix** should be prepared in a sterile vial **multiplying** each single volume by the number **N** of samples (incl. controls). *Additionally, 10% more volume should be calculated for reasons of inaccuracy*. The reagents should be pipetted in same order as indicated in the table:

Reagent	Volume	Master Mix Volume
Detection Mix (yellow)	**5 µl**	**5 µl** x (N + 10%)
Detection Mix (white)	**5 µl**	**5 µl** x (N + 10%)
Water (green)	**0,5 µl**	**0,5 µl** x (N + 10%)
Enzyme Mix *ready to use* (blue)	**12,5 µl**	**12,5 µl** x (N + 10%)

Mix prepared master mix well by gently pipetting (about **15 – 20 x, do not vortex**) and aliquot **23 µl** into each PCR reaction tube.

Samples

Add **2 µl** of each sample DNA in the corresponding PCR reaction tube; use first **both controls** (1. negative control, 2 µl and 2. positive control, 2 µl). Close the tubes and transfer them into the real time PCR instrument (keep position of samples).

Protocol

Activate following **PCR-protocol** and perform subsequently the *real time* PCR:

Experimental Protocol

Program:	**Denaturation**		1
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	94	120	None

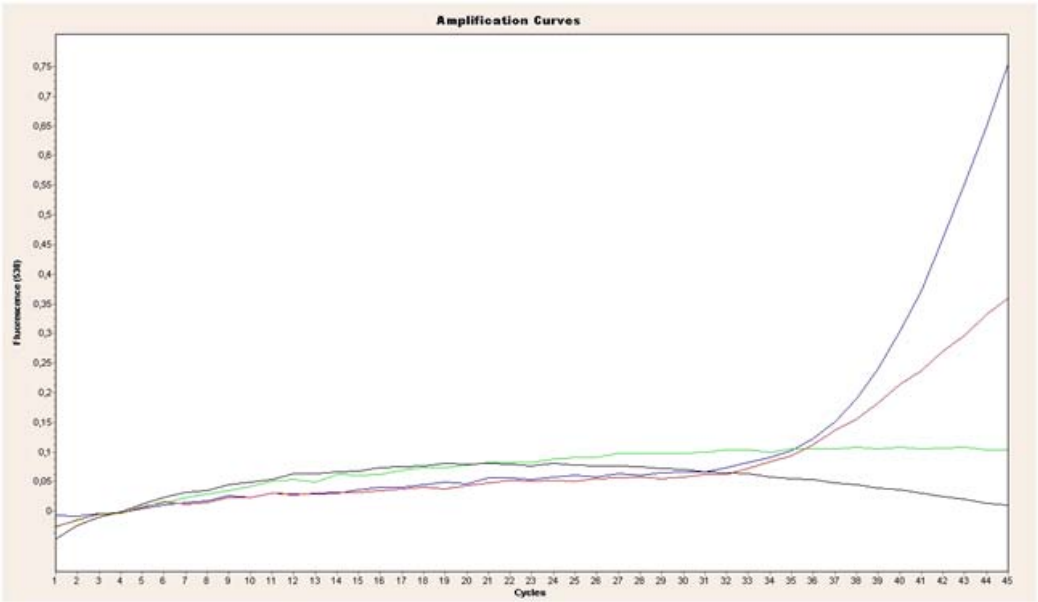
Program:	**Amplifikation**		45
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	94	30	None
2	64	60	**Single**
Program:	**Cooling**		1
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	40	30	None

9 Analysis of Genotype and Interpretation of Results

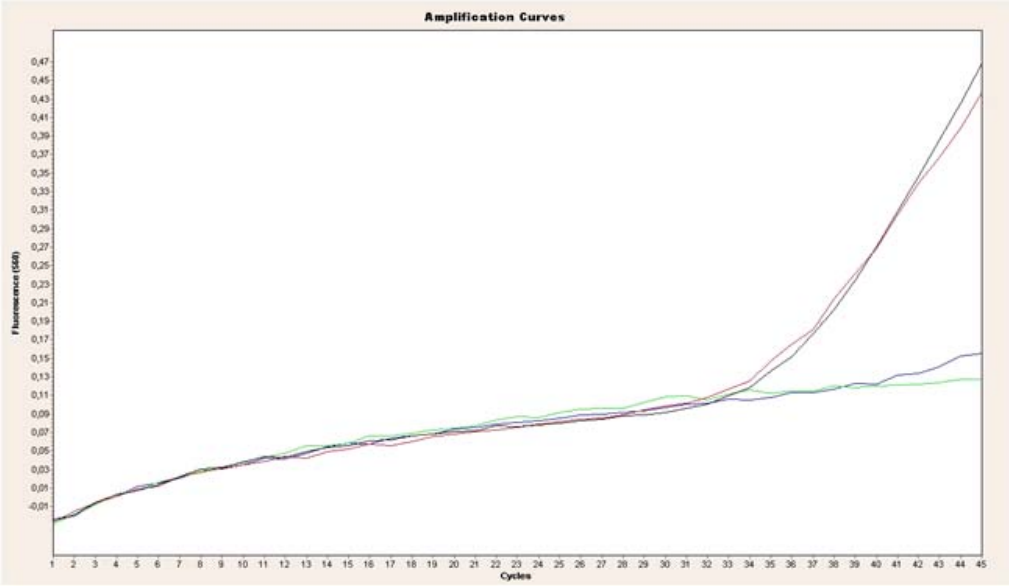
Results of the analysis for the G20210A polymorphism are shown for at **510 - 530 nm / green** and **550 - 560 nm / yellow** (choose corresponding channel of your real time PCR instrument). The provided Positive Control contains a template which is heterozygous for the G20210A-polymorphism (one allele carries the mutation, the other is wild type).

Following **figures** shows typical **examples** for **homozygous** as well as **heterozygous** samples on the LightCycler 2.0. Use a appropriate color compensation file, if necessary e.g. LightCycler.

G-Allele at 530 nm



A-Allele at 560 nm



10 Troubleshooting

No fluorescence peak with positive control or samples at about **510-530 nm or 550-560 nm:**

- Proof PCR-program of the real time PCR instrument in use:

⇒ repeat analysis with corrected protocol.

- MutaPLATE® Factor II (TM) kit was thawed/ frozen more than twice or stored longer than four days at 2-8 °C:

⇒ consider storage recommendations. Repeat analysis with new MutaPLATE® Factor II (TM) reagents.

- low quality of DNA -template:

⇒ exactly follow the manufacturer`s manual for DNA extraction.

Low fluorescence peak at about **510 - 530 nm or 550 - 560 nm**:

- mix single components carfully before use (only by pipetting several times - do not vortex!).
- cool all stock solutions during the working steps in suited manner and protect the detection mix from light.
- Working on ice or with cooled (4°C) Block is recommended.

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