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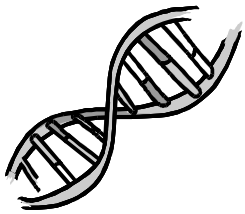
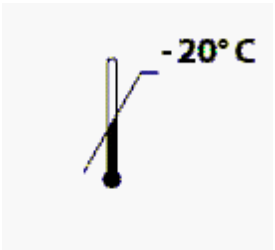


MutaPLATE® Factor V (TM)

(TAQ-Man) real time PCR Kit

PCR test for analysis of the G1691A mutation in the Factor V gene 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 KF1901732 Σ **32**

REF KF1901796 Σ **96**

Table of Content

1 Intended Use

MutaPLATE ® Factor V (TAQ-Man) *real time* PCR kit is a molecular biological test for the detection G1691A mutation in the Factor V gene. 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

Thromboses are connected with disorders of the human coagulation system and can even cause symptoms as serious as pulmonary embolism. One of the factors regulating the coagulation system is the activated protein C (APC), a serine protease which, together with a co-factor, influences the clotting process by the proteolytic inactivation of two components of the coagulation cascade, factors Va and VIIIa. Factor Va is developed by splitting from the factor V protein, a process brought about by the factor IIa.

According to the present state of knowledge, the APC resistance is the most frequent genetic risk factor for venous clotting disorders. This is caused by a point mutation in the factor V gene, in which at position 1691 a guanine (G) is replaced by an adenine (A). As a result of this, the 506th amino acid (arginine) is replaced by glutamine in the translated protein (FV:Q506). The factor V genetic product, changed in this way, is then described as the Factor V-Leiden (FVL). Because the mutated amino acid is localized with the APC protein in the combining site, the FVL variant cannot be sufficiently splitted from the APC and inactivated. This leads to an accumulation of the factor Va, resulting in a raised tendency to coagulation, which in its turn causes a higher risk of thrombosis.

Approximately 5% of the population have a higher APC resistance, and the rate in patients with a personal or familial previous history of thrombosis is 20-60%. The allelic frequency of the mutation described is therefore ten times higher than in all other genetic risk factors of thrombosis, such as a deficiency of protein C or anti thrombin. Homozygous patients with these characteristics have a 50-100 times greater risk of thrombosis, this risk is in heterozygous patients 5-10 times greater. In combination with other risks, the probability of suffering from thrombosis rises further.

For example, oral contraceptives lead to a five times greater risk of thrombosis, this risk in combination with a heterozygous factor V mutation is then 35 times greater.

Measuring the APC resistance is an important laboratorial diagnostic parameter in identifying functional disorders of the coagulation system, and can point to the presence of a FVL genotype. In order to ascertain the genotype exactly, especially to differentiate between heterozygous and homozygous, an analysis at DNA level is necessary.

Variation	RsNumber	Effect		
		Wildtype	Heterozygous	Homozygous
Arg506Gln/ G1691A	rs6025	Normal inactivation of FV by activated protein C. No increased risk of venous thrombosis.	7-fold increased risk of venous thrombosis.	Up to 100-fold increased risk for venous thrombosis. The FV Leiden mutation affects one of the target sites for APC-catalyzed inactivation of activated FV. This defect leads to a reduced anticoagulant effect of APC and thereby to an increased tendency to thrombosis.

[1] J. P. Vandenbroucke, T. Koster, E. Briët, P. H. Reitsma, R. M. Bertina und F. R. Rosendaal, „Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation.," *Lancet,* Bd. 344, Nr. 8935, pp. 1453-1457, 1994.

[2] P. A. Kyrle, F. R. Rosendaal und S. Eichinger, „Risk assessment for recurrent venous thrombosis.," *Lancet,* Bd. 376, Nr. 9757, pp. 2032-2039, 2010.

[3] G. A. Nicolaes und B. Dahlbäck, „Factor V and thrombotic disease: description of a janus-faced protein.," *Arterioscler Thromb Vasc Biol,* Bd. 22, Nr. 4, pp. 530-538, 2002.

[4] G. Lippi, E. J. Favaloro, M. Montagnana, F. Manzato, G. C. Guidi und M. Franchini, „Inherited and acquired factor V deficiency.," *Blood Coagul Fibrinolysis,* Bd. 22, Nr. 3, pp. 160-166, 2011.

[5] F. R. Rosendaal, D. S. Siscovick, S. M. Schwartz, R. K. Beverly, B. M. Psaty, W. T. Longstreth, T. E. Raghunathan, T. D. Koepsell und P. H. Reitsma, „Factor V Leiden (resistance to activated protein C) increases the risk of myocardial infarction in young women.," *Blood,* Bd. 89, Nr. 8, pp. 2817-2821, 1997.

3 Principle of the Test

MutaPLATE® Factor V (TAQ-Man) *real time* PCR Kit contains specific primers and additional material for the detection of the G1691A polymorphism of the factor V gene. The variable area of the 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	Enzyme Mix	435 µl	3 x 435 µl
Yellow	Detection Mix **G - Allele**	175 µl	3 x 175 µl
White	Detection Mix **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

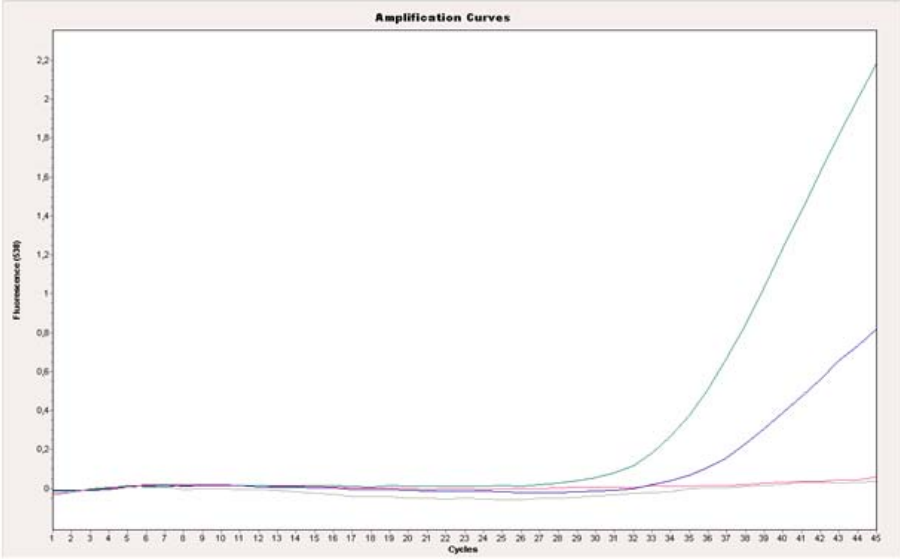
Program:	**Amplifikation**		45
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	94	30	None
2	62	60	**Single**
Program:	**Cooling**		1
Segment Number	Temperature Target (°C)	Hold Time (sec)	Acquisition Mode
1	40	30	None

9 Analysis of Genotype and Interpretation of Results

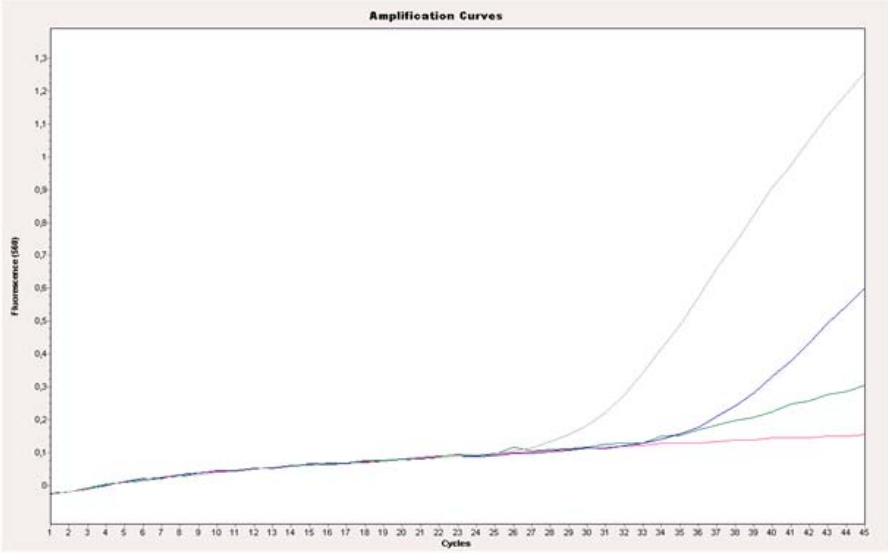
Results of the analysis for the G1691A polymorphism are shown for at **510 - 530 nm / green** and **550 - 560 nm / yellow** (choose corresponding channel of your real time PCR instrument). The provided Positive Control contains a template which is heterozygous for the G1691A polymorphism (one allele 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 V (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 V (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 carefully 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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