GenVinSet

Factor V G1691A

Instructions for Use

Kit for detection of Factor V G1691A mutation

For In Vitro diagnostic use

Prodcut code GVS-FV-24 (24 tests)
GVS-FV-48 (48 tests)

Store from –18 to –30ºC

CE

Rev06 / 2020-04-15

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Intended use

GENVINSET® Factor V G1691A is a kit for the determination of the G>A transition in the 1691 position, located in exon 10 of the Factor V gene, using Real Time PCR technology with specific TaqMan® probes.

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Summary and explanation

Factor V (FV) is a protein that plays an important role in the coagulation cascade. It is converted into its active form (FVa) by action of thrombin. Then, FVa in combination with Factor X activated (FXa), constitutes a complex with protease activity which catalyses the conversion of prothrombin (Factor II) into thrombin. (1). Thrombin ats upon fibrinogen producing a dense network of fibrin, which is the main component of a blood clot. The FV gene is located on the chromosome 1q23, spans 70 kb.

The inactive form of FV circulates with little/no procoagulant activity. One anticoagulation mechanism involves the performance of the activated protein C (APC), which inactivates FV, consequently blocking the production of thrombin. Factor V Leiden (FLV) is the mutated form of FV. Its detection is one of the most frequently performed genetic tests (2, 3). The mutation that occurs in the Factor V gene consists of a single nucleotide polymorphism (SNP) in position 1691 within exon 10. The aforementioned mutation, known as G1691A, results in a transition from a guanine (G) into an adenine (A). As a result, the amino acid located on position 506, becomes a glutamine, instead of an arginine, which reduces the APC affinity for the FVa, preventing its degradation. This decrease in the APC affinity for FV is known as APC resistance and prevents the activation of FV which, in turn, produces an accumulation of thrombin in blood, leading to an increased risk of blood clotting. APC resistance is regarded as the most prevalent coagulation abnormality associated with venous thrombosis (4). The FVL mutation is responsible for 90–95% of APC resistant cases (5).

The FVL disorder is an autosomal dominant trait that displays incomplete dominance. The prevalence of the mutation ranges from 1 to 13% and varies among the different populations (6). Homozygotes and heterozygotes for the FVL mutation have an 80-fold and 8-fold increased risk of developing venous thrombosis, respectively (7, 8). Deep vein thrombosis in the heart or lung may cause pulmonary embolisms or transient ischemic attacks (mini-strokes) (9).

The FVL mutation in combination with other clotting risk factors, including smoking, hormonal contraception, estrogen hormone replacement, and recent surgery, further increases the risk for venous thromboembolism and stroke (10, 11). Women with a FVL mutation have an increased risk of miscarriage and stillbirth due to the clot formation in the umbilical chord, placenta or fetus (12). Moreover, the hypercoagulation state caused by the FLV mutation is associated with retinal artery occlusion, one of the most common ophthalmological diseases (13).

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The confirmation of the FLV mutation allows to design the appropriate protocols and treatments for indivuduals and families at risk of future venous thromboembolism.

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Procedure principles

The detection method used by GENVINSET® Factor V G1691A is based on the Real Time PCR technology, using TaqMan® probes that specifically anneal to position 1691 of the exon 10 of the Factor V gene, monitoring the presence of A and/or G nucleotides.

This technique provides high resolution, high sensitivity, specificity and reproducibility.

(*) See Section "Procedure Limitations".

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Kit contents

GVS-FV-24 (24 tests)

- GVS-FV-PM: Primer Mix (PM)
- GVS-FV-MM: Master Mix (MM)
- GVS-FV-C1: Control WT/WT (C1)
- GVS-FV-C2: Control MUT/MUT (C2)
- GVS-RB: Reaction Blank (RB)

GVS-FV-48 (48 tests)

- GVS-FV-PM: Primer Mix (PM)
- GVS-FV-MM: Master Mix (MM)
- GVS-FV-C1: Control WT/WT (C1)
- GVS-FV-C2: Control MUT/MUT (C2)
- GVS-RB: Reaction Blank (RB)

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Kit storage

In order to ensure a proper performance, reagents should be stored from -18ºC to -30ºC until their expiration date, indicated on the label of the vial. Do not perform more than 3 freeze/thaw cycles to the Primer Mix (GVS-FV-PM) and Master Mix (GVS-FV-MM) vials, as this could decrease the sensitivity of the assay and impair results.

Due to the photosensitivity nature of the reagents, avoid continous exposure to light.

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Materials required but not supplied

General

- Gloves
- Lab coat

Consumables

- Filter tips (P1000, P200 & P10)
- 1.5 ml autoclaved tubes
- q-PCR instrument specific reagents (in the case of using RotorGene Q, only 0.1 ml tubes are allowed)

Equipment

- q-PCR instrument. The following devices have been validated:
  - StepOne™, Applied Biosystems™
  - 7500 Real-Time PCR System, Applied Biosystems™
  - LightCycler® 96 System, Roche
  - LightCycler® 480, Roche
  - Rotor-Gene® Q, Qiagen®
- Vortex mixer
- Pipettes (P1000, P200 & P10)

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Sample collection and preparation

The present test should only be performed with complete blood samples treated with EDTA anti coagulation agents or citrate. Heparin can interfere with the PCR process and should not be used in this procedure.

The technique is compatible with several DNA extraction methods. Before delivering results with a diagnostic purpose, a validation assay with such extraction method should be done.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, the corresponding basic (universal) precautions should be taken. Samples should always be handled wearing the appropriate personal protection equipment.

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Usage procedures

A) PCR setup

Precautions

- Thaw all of the kit components before starting the assay, mix and centrifuge them.
- Work on ice or over a cool block.
- The PCR should be setup in the pre-PCR area.
- Use only filtered tips and 1.5 ml autoclaved tubes.
- Use gloves and lab coat at all times.
- In each test performed it is recommended to use one control sample for each G/A variant.

1. Thaw the samples. Prepare a mix with the Master Mix and the Primer Mix for n+1 samples:

	Vol. per sample (µL)
Master Mix	5
Primer Mix	4

2. Pipette 9 µL of this mix into the PCR tubes and add 1µL of DNA or Reaction Blank in case of the contamination control well.

3. Seal the plate with convenient sealer and centrifuge 1 min at 360 xg to ensure that all the volume settles to the bottom of the tube.

4. Place the plate in the thermal cycler and start the following program.

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B) Thermal cycler configuration

1. Set up the following amplification program:

	Cycle number	Temperature (ºC)	Time (mm:ss)	Ramp (%)	
Denaturalization	1	95	05:00	100	X
Cycles	50	95	00:15	100	X
		62	1:00	100	Single
Cooling	1	15	∞	100	X

2. Set up the reading channels.

The emitted fluorescence must be read in FAM (495-520 nm) and HEX (535-554 nm) channels.

NOTE – Special settings for Rotor Gene Q:

a. Open the Rotor-Gene Q – Pure Detection software. Select the tab "Advanced" in the window "New Run", and press "New".

b. Select the type of rotor used (only 0.1 ml tubes accepted, see section "Material required but not supplied"). Select the "Locking Ring Attached" box and continue by clicking on "Next".

c. Set the "Reaction Volume" at 10 µl, and identify the operator and the samples.

d. Click on "Edit Profile" and set up the amplification program detailed above. Select the step 60 sec at 62 ºC, and click on "Acquiring to Cycling A". Set "Green" and "Yellow" as the fluorescence acquisition channels. Press "OK". Click on "OK" to accept and close the "Edit Profile" window.

e. Click on "Gain Optimisation" in the "Run New Wizard" dialog box to open the "Auto-Gain Optimization Setup" window. Scroll down the "Channel Settings" menu and select "Acquiring Channels". Then click on "Add". In the "Auto-Gain Optimisation Channel Settings" window", set the following parameters for each channel ("Green" and "Yellow"):

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Tube position = 1

Target Sample Range: 5 FI up to 10 FI

Acceptable Gain Range: -10 to 10

f. Check the box "Perform Optimisation Before 1st Acquisition", and click on "Close".

g. Select "Next" and then "Start Run" in the "New Run Wizard" window.

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Results

GENVINSET® Factor V G1691A constitutes a qualitative technique to detect the presence of a G and/or an A on the 1691 position of the exon 10 of the Factor V gene.

It is not necessary to select any passive reference.

Using the present kit, the following results can be obtained:

Detection of A at position 1691

In the FAM channel, the following amplification plot can be observed:

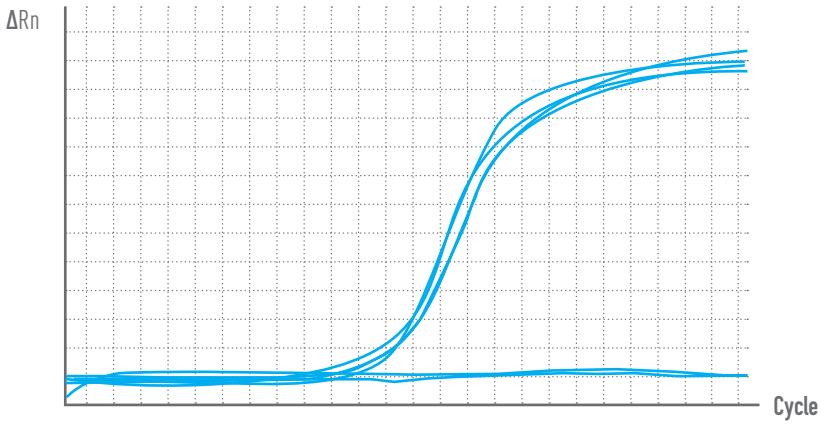

Figure 1. Positive and negative samples in the FAM channel.

Those samples that report an amplification curve can be considered as G1691A A positive and they are identified by a numeric value called "Crossing Point" (Cp). This value corresponds to the cycle in which fluorescence is detected and, thereof, the amplification can be considered as positive.

Detection of G at position 1691

In the VIC/HEX channel, the following amplification plot can be observed:

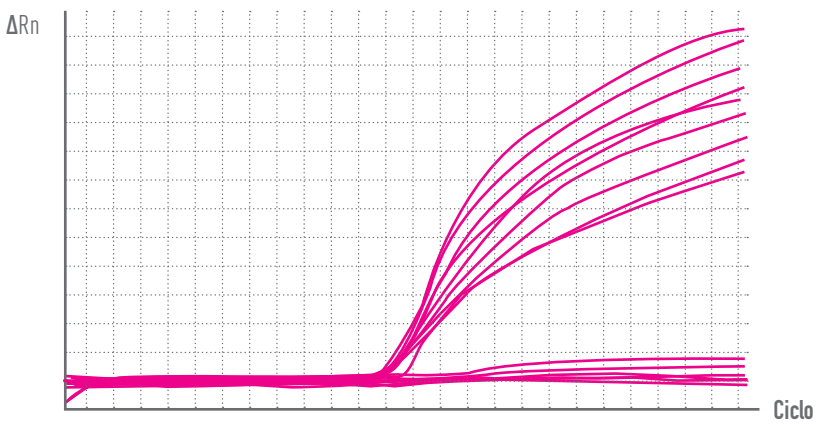

Figure 2. Positive and negative samples in the VIC/HEX channel

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Those samples that report an amplification curve can be considered as G1691A G positive.

Genotyping analysis

In "Genotyping" or "Allelic Discrimination" analysis types, select FAM channel (mutation) on the Y axis, and HEX channel (wildtype) on the X axis. Results will appear similarly as in Figure 2, in which each dot consists of an X and Y component. The different kind of samples, wt/wt, wt/mut and mut/mut, will be distributed into 3 groups within the plot. The Reaction Blank will be placed near the origin of coordinates (0,0).

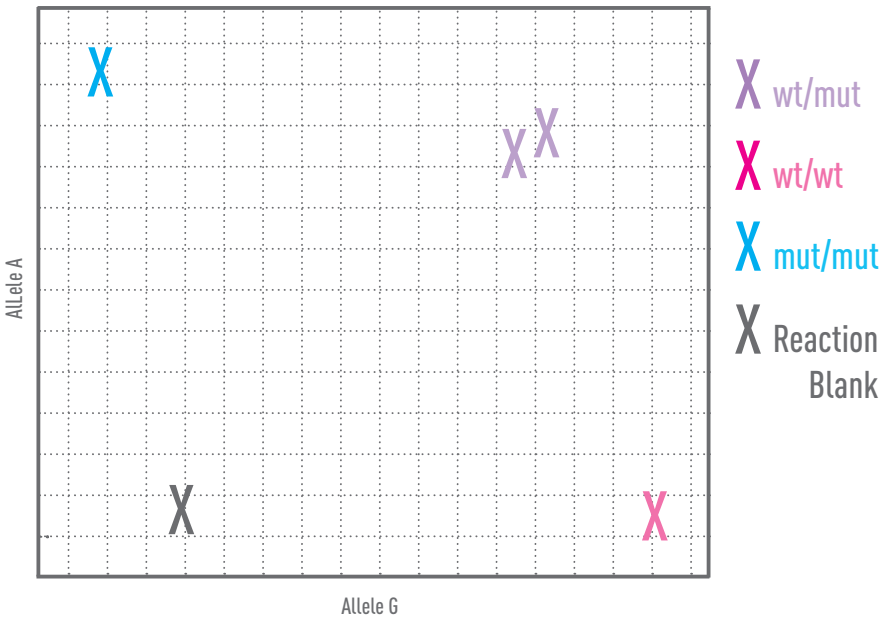

Figure 3. Plot showing two heterozygous samples (wt/mut), one wild type sample (wt/wt), one homozygous mutated sample (mut/mut) for G1691A mutation, and the control of contamination (RB), using Genvinset® Factor V G1691A kit. Graphic distribution according to detected alleles.

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Quality control

Due to the qualitative nature of this test, it will not be necessary to perform a calibration.

The following criteria have to be taken into account in order to validate an assay:

- The Reaction Blank should report negative results in both FAM and VIC/HEX channels. An amplification curve with a Cp>35 value should be considered as negative. A Cp<35 value is associated with a contamination in the Reaction Blank and, therefore, all results from the experiment should be discarded.
- An heterozygous sample should report positive results in both FAM and VIC/HEX channels.

The assay must be performed according to the kits recommendations, as well as other quality control procedures that comply with local, federal and/or certifying agencies specifications.

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Specific operation data

1. Analytical specificity

The probes align specifically on the 1691 position at the exon 10 of the Factor V gene. No unspecific alignments have been detected.

2. Analytical sensitivity

A dilution assay was performed, using 1:4 serial dilutions of a wild type DNA sample for G1691A mutation (no mutated alleles), one heterozygote (one mutated allele) and another one homozygote (two mutated alleles), obtained by a conventional extraction system, with an initial concentration of 92.1, 49.4 and 37.0 ng/µL respectively. The following analytical sensitivity results were obtained:

- Detection Limit of wild type and mutated allele = 0.20 ng/µl (*)

(*) Cp < 35

3. Diagnostic sensitivity and specificity

In a human genomic DNA study, 89 samples obtained from several laboratories were analyzed. They were previously genotyped by another commercial kit.

All the tested samples were validated. The following results were obtained:

GENVINSET® Factor V G1691A		G/G	G/A	A/A
Previous method	G/G	75	0	0
	G/A	0	11	0
	A/A	0	0	3

There is a 100% match in the results obtained with GENVINSET® Factor V G1691A and the genotyping previously obtained with another commercial kit.

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Procedure limitations

- The method detects the G/A SNP at position 1691 of the exon 10 of the Factor V gene.
- Mutations or polymorphisms at annealing primer/probe sites are possible and may result of the lack of allele definition. Other technologies could be necessaries to resolve the typing.
- All the aforementioned conditions for the setup of the PCR should be carefully controlled. Any performances that do not meet such indications, can lead to poor results.
- All GENVINSET® reagents manipulation must be done according to the general good practises of laboratory, being adapted to local regulations.
- The q-PCR thermal cycler must be calibrated according to the manufacturers´ recommendations and should be used accordingly to the manufacturer's instructions.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiration date.
- Do not use the kit if there are any suspicions of possible loss of reactivity, contamination, external box deterioration or any other incidence that might affect the kits performance.
- Data and result interpretation should be revised by qualified personnel.
- Discard expired reagents according to applicable regulations.

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Troubleshooting guide

Problem

- **Probable cause(s)**
  - Suggested corrective measure(s)

Reaction Blank control (H_2O) is positive

- **Primer Mix/Master Mix/Reaction Blank contamination**
  - Repeat the experiment with new Primer Mix/Master Mix/ Reaction Blank aliquots
  - Handle the kit components always according to accepted lab practices in oirder to avoid contamination
  - Verify manipulation and storage conditions
  - Discard contaminated reagents
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the pre-PCR area have been followed
  - Check for possible contamination problems in other PCR techniques
  - Confirm suitability of the used reagents (1.5 ml tubes, pipette tips)
- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet

Low or no signal in all samples. Control samples are OK

- **Bad quality of DNA**
  - Repeat the sample extraction verifying each step (hemoglobin can interfere with the PCR)
- **Blood processed without previous frozen step**
  - Repeat extraction with a new blood aliquot previously frozen
- **Samples with very low DNA concentration**
  - Check DNA concentration
- **DNA samples with high concentration**
  - Perform the assay using diluted samples

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Fluorescence intensity too low

- **Kit degradation**
  - Check that the storage of the kit is correct, reviewing both proper temperature conditions and light exposure (which should be avoid
  - Avoid more than 3 freeze/thaw cycles of the Primer Mix vial
  - Aliquote the reagents if necessary
  - Repeat the test with new reagents

Control C1 (DNA sample) is positive

- **Cross contamination**
  - Always handle the kit components following all necessary practices to avoid contamination
- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet

Control C2 is negative

- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet

Fluorescence intensity varies

- **The dirtiness on the outside of the tube walls interferes with the signal**
  - Handle all consumables wearing gloves
- **Volume is not settled to the bottom of the well or there are bubbles**
  - Perform a brief centrifugation to ensure that the volume settles to the bottom of the well and to remove all bubbles
- **Pipetting error**
  - Check that the correct volume has been added

There is no fluorescence signal

- **Incorrect reading channels selected**
  - Set the appropriate reading channels
- **Pipetting error or reagent absence**
  - Control the pipetting and the reaction setup configuration
  - Repeat the PCR
- **No reading channel was selected in the thermal cycler's program.**
  - Check and modify the thermal cycler program if necessary

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Changes to version 05

Version	Description of the modification
Rev. 06	Modification of PCR annealing temperature, from 64ºC o 62ºC.

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Explanation of symbols used on the labels

For in vitro diagnostic use

This product fulfills the requirements of Directive 98/79/EC on in vitro diagnostic medical device

Catalogue number

Lot number

Expiration date

Contents sufficient for <n> tests

Manufactured by

Store at

Keep away from sunlight

Positive control

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