GenVinSet

Factor II G20210A

Instructions for Use

Kit for detection of prothrombin (FII) G20210A mutation

For In Vitro diagnostic use

Product code GVS-FII-24 (24 tests)
GVS-FII-48 (48 tests)

Store from –18 to –30ºC

Rev05 / 2020-04-15

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Factor II
G20210A

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Intended use

GENVINSET® Factor II G20210A is a kit for the determination of the G>A transition in the 20210 position, located in the 3'UTR of the prothrombin gen, using Real Time PCR technology with specific TaqMan® probes.

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Summary and explanation

Prothrombin is an essential component of the blood-clotting mechanism. It is a glycoprotein generated by the liver and present in blood plasma. Prothrombin undergoes a proteolitic conversion to thrombin, catalysed by a clotting factor, known as Factor X or prothrombinase. Thrombin, in turn, catalyses the conversion of fibrinogen, also present in plasma, into fibrin. Fibrin, together with platelets, forms a clot during the coagulation process. Under normal circumstances, prothrombin is conversed into thrombin only when a tissue suffers an injury; therefore, fribrin and blood clots are only formed as a response to bleeding.

Prothrombin gene is composed of 14 exons, spans about 21 kb (1) and is located in chromosome 11 (2). This gene encondes a 622 aminoacid propeptide, with a molecular mass of approximately 70 kD. The prothrombin protein undergoes several cleavage events to generate the active thrombin protein, which is composed of a light (alpha) and heavy (beta) chain covalently linked by a disulfide bond.

The regulation of prothrombin expression is crucial to mantain a normal hemostatic function. Mutations that cause a lower level of prothrombin or alter its function are uncommon but are typically associated to a severe hemorrhagic phenotype. In contrast, the G20210A mutation, which occurs in the 3'UTR of the prothrombin gene causes a higher expression of the protein and shows a frequency of over 4% in some populations (4). This mutation consists of a G>A transition in the poly(A) tail of the messenger RNA (5). Heterozygous individuals for the G20210A gene variant present, on average, a 25% increase in the expression levels of the protein. The G20210A mutation is physiologycally relevant, since it is associated to a significant increase of venous (1,6) and arterial thromboembolism (7-9) in heterozygous individuals. The effects can become particularly severe in some organs and subpopulations (10-12).

This mutation shows a high prevalence (18%) in families with thrombosis and in patients with no familial antecedents (6.2%) (13).

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Procedure principles

The detection method used by GENVINSET® Factor II G20210A is based on the Real Time PCR technology, using TaqMan® probes that specifically anneal to position 20210 of the 3'UTR of the prothrombin 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-FII-24 (24 tests)

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

GVS-FII-48 (48 tests)

- GVS-FII-PM: Primer Mix (PM)
- GVS-FII-MM: Master Mix (MM)
- GVS-FII-C1: Control WT/WT (C1)
- GVS-FII-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-FII-PM) and Master Mix (GVS-FII-MM) vials, as this could decrease the sensitivity of the assay and impair results.

Due to the photosensitivity nature of the reagents, avoid continuous 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 (%)	Analysis
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 click "New".

b. Select the type of rotor used (only 0.1 ml tubes accepted, see section 6). Select the "Locking Ring Attached" box and continue by clicking "Next".

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

d. Click on "Edit Profile" and set up the amplification program (see section 8.C.1). 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 window "Auto-Gain Optimisation Channel Settings", 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 II G20210A constitutes a qualitative technique to detect the presence of a G and/or an A on the 20210 position of the 3'UTR of the prothrombin 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 20210

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

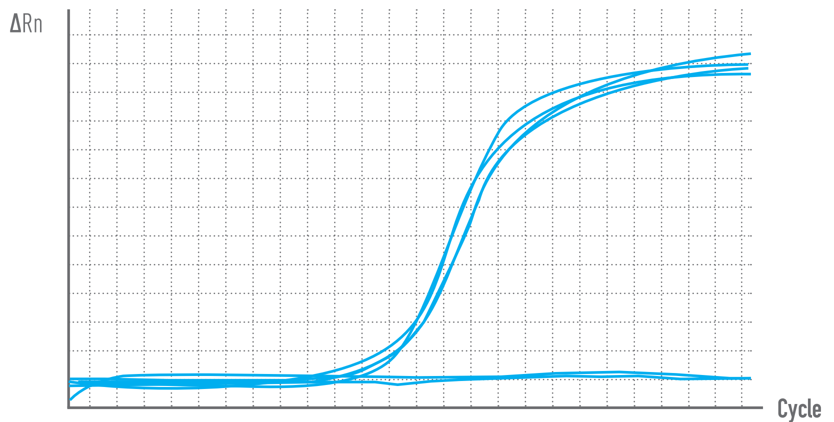

Figure 1. Positive and negative samples in the FAM channel.

Those samples that report an amplification curve can be considered as G20210A 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 20210

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

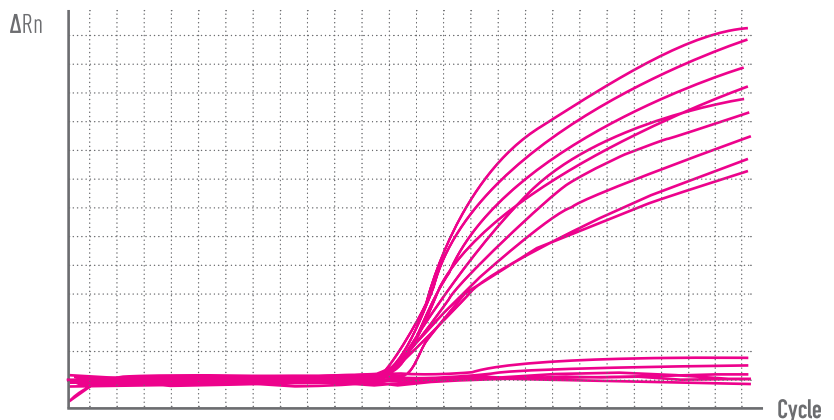

Figure 2. Positive and negative samples in the VIC/HEX channel

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Those samples that report an amplification curve can be considered as G20210A 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).

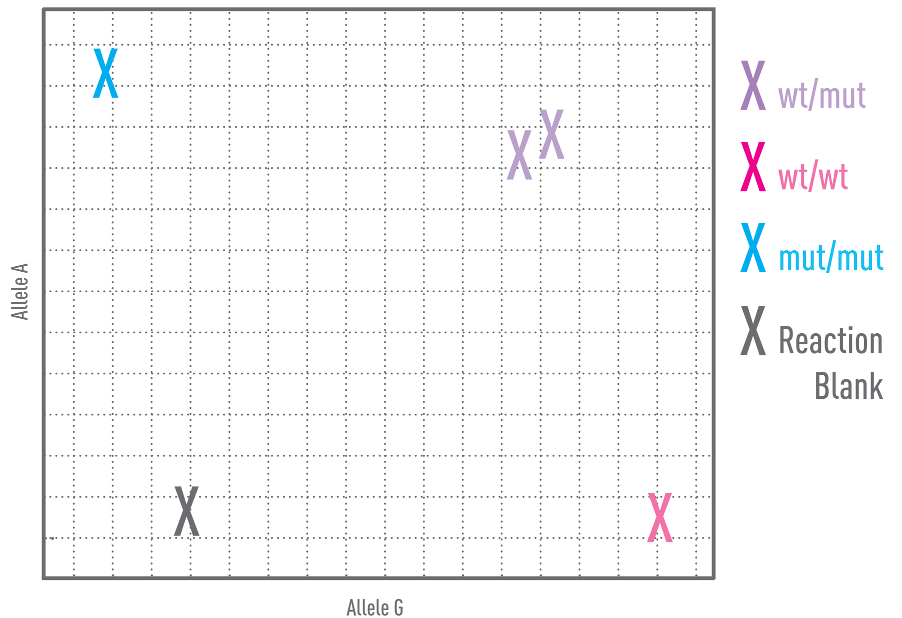

Figure 3. Plot showing two heterozygous samples (wt/mut), one wild type sample (wt/wt), one homozygous mutated sample (mut/mut) for G20210A mutation, and the control of contamination (RB), using Genvinset® Factor II G20210A kit.

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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 kit 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 20210 position at the 3'UTR of the prothrombin gene. No unspecific alignments have been detected. No cross-reaction phenomena with genomic DNA have been reported.

2. Analytical sensitivity

A dilution assay was performed, using 1:4 serial dilutions of a wild type sample (no mutated alleles) and an heterozygous sample (one mutated allele), obtained by a conventional extraction system, at an initial concentration of 52.5, 54.8 and 83.7 ng/μL, respectively. The following analytical sensitivity results were obtained:

- Detection Limit of wild type and mutated allele = 0.25 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 II G20210A		G/G	G/A	A/A
Previous method	G/G	75	0	0
	G/A	0	13	0
	A/A	0	0	1

There is a 100% match in the results obtained with GENVINSET® Factor II G20210A and the genotyping previously obtained with another commercial kit.

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Procedure limitations

- The method detects the G/A SNP at position 20210 of the 3´UTR of the prothrombin 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.
- Dircard expired reagents according to applicable regulations.

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Troubleshooting guide

Problem

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

Reaction Blank (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 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
    - Repeat the PCR
- No reading channel was selected in the thermal cycler program.
    - Check and modify the thermal cycler program if necessary

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References

1. Degen, S. J. F., Davie, E. W. Nucleotide sequence of the gene for human prothrombin.Biochemistry 26: 6165-6177, 1987

2. Royle, N. J., Irwin, D. M., Koschinsky, M. L., MacGillivray, R. T. A., Hamerton, J. L.Human genes encoding prothrombin and ceruloplasmin map to 11p11-q12 and 3q21-24, respectively.Somat. Cell Molec. Genet. 13: 285-292, 1987.

3. Poort S, Rosendaal F, Reitsma P, Bertina R. 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;88: 3698-3703.

4. Rosendaal F, Doggen C, Zivelin A, et al. Geographic distribution of the 20210 G to A prothrombin variant. Thromb Haemost. 1998;79:706-708.

5. Degan S, MacGillivray R, Davie E. Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin. Biochemistry. 1983;22:2087-2097.

6. Soria J, Almasy L, Souto J, et al. Linkage analysis demonstrates that the prothrombotic G20210A mutation jointly influences plasma prothrombin levels and risk of thrombosis. Blood. 2000;95: 2780-2785.

7. Franco R, Trip M, ten Cate H, et al. The 20210 G A mutation in the 3-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. Br J Haematol. 1999;104:50-54.

8. Watzke H, Schuttrumpf J, Graf S, Huber K, Panzer S. Increased prevalence of polymorphism in the gene coding for human prothrombin in patients with coronary heart disease. Thromb Res. 1997;87:521-526.

9. Burzotta F, Paciaroni K, DeStefano V, et al. Increased prevalence of the G20210A prothrombin gene variant in adult coronary syndromes without metabolic or acquired risk factors or with limited extent of disease. Eur Heart J. 2002;23:26-30.

10. Rosendaal F, Siscovick D, Schwartz S, Psaty B, Raghunathan T, Vos H. A common prothrombin variant (20210 G to A) increases the risk of myocardial infarction in young women. Blood. 1997; 90:1747-1750.

11. Kupferminc M, Peri H, Zwang E, Yaron Y, Wolman I, Eldor A. High prevalence of the prothrombin gene mutation in women with interuterine growth retardation, abruptio placentae, and second trimester loss. Acta Obstet Gynecol Scand. 2000;79:963-967.

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12. Mercier E, Quere I, Campello C, Mares P, Gris J. The 20210A allele of the prothrombin gene is frequent in young women with unexplained spinal cord infarction. Blood. 1998;92:1840-1841.

13. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A commongenetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 88, 1996, 3698-3703.

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Changes to version 04

Version	Description of the modification
Rev. 05	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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