Genvinset®

Factor V G1691A

INSTRUCTIONS FOR USE

Kit for detection of Factor V G1691A mutation

For In Vitro Diagnostic use

Rev. 08 / 2022-05-27

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CE

Product codes:	UDI-DI:	Store
GVS-FV-24 (24 tests)	8437016942376	from -30°C to -18°C
GVS-FV-48 (48 tests)	8437016942383	
GVS-FV-96 (96 tests)	8437016942703	

Genvinset®

Factor V G1691A

Index

1- Information for safety

Please, read completely these instructions for use and follow them during the use of the current IVD kit.

The IVD kit shall be used by experts with strong experience in DNA analysis and interpretation of genetic results.

Consult the manufacturer if there are doubts involving the description of the assay method. Contact by phone +34 976 094 603 or email address *customersupport@bdrdiagnostics.com*.

The IVD kit has a limited shelf life. Make sure that the shelf life is not expired before using the kit. Reagents from a kit beyond its expiry date might be degraded, which could impair results. Discard the expired reagents following applicable regulations.

The contamination of the sample or the reagent can render incorrect results. Be careful during the DNA extraction and sample and reagents manipulation.

This kit can be damaged during transport or storage. Do not use the kit in case of suspected damage during transport. Follow carefully the storage conditions described in the label and the IFU.

Ensure that the waste is managed according to local regulations. Incorrect waste management can result in environmental contamination.

The toxicological properties of the kit have not been studied in-depth, so it is recommended to avoid contact with skin and mucous membranes. Do not ingest. Manual Safety Data Sheets (MSDS) are available for the customers by request.

Ensure that this kit is adequate for the requested analysis by the clinician.

2- Intended Use

Genvinset® Factor V G1691A is a semi-automated kit for the *in vitro* qualitative detection of the G1691A mutation (NCBI dbSNP rs6025; NM_000130.5:c.1601G>A) in the factor V (*FV*) gene (OMIM: 612309) associated with thrombophilia risk, in genomic DNA extracted from whole blood using Real Time PCR technology with specific TaqMan® probes.

Patients who can benefit from this determination are those referred by a specialist. The results of this test should not be the only ones on which the therapeutic decision is based and should be used as an aid in the diagnosis together with results of other markers of the disease.

The intended user of the kit is technical personnel trained to carry out the protocol and the interpretation of results described in this document.

3- Summary and explanation

Thrombophilia consists in the predisposition to form blood clots, caused by an underlying hypercoagulation state attributable to inherited or acquired disorders of blood coagulation or fibrinolysis.

It is well documented that the G1691A mutation (NCBI dbSNP rs6025; NM_000130.5:c.1601G>A) in the gene that encodes for the coagulation Factor V (known as FV Leiden or FVL) is associated with an increased risk of thrombophilia and venous thrombosis[1,2].

The molecular basis of FV Leiden is a missense mutation at position G1691A, which results in a substitution of arginine to glutamine (R506Q) on one of the activated protein C (APC) cleavage sites. As a result, activated Factor V becomes resistant to cleavage by APC and its inactivation rate becomes 10-fold slower, thus maintaining its procoagulant activity[3–5].

FVL mutation is inherited in an autosomal-dominant fashion, and it is the most prevalent risk factor for thrombosis in the Caucasian population, being present in 3-7% of individuals. Conversely, it is rare among Asians and Africans[6]. Heterozygous carriers of FVL have an approximately 3- to 5-fold increased risk of venous thromboembolism (VTE), whereas the risk in homozygous carriers is estimated to be increased 80 times[7].

The absolute incidence of VTE in patients with FVL mutation ranges from 0.19% per year to 0.45% per year, compared to 0.10% per year in individuals without the mutation[8]. Vice versa, FVL is present in the heterozygous form in approximately 15–20% of VTE patients[9]. However, not all carriers of the FVL mutation develop VTE and their absolute thrombotic risk depends on the interaction between this and other inherited thrombophilia mutations, or acquired high-risk factors, such as age, pregnancy, immobilization, long travel times, surgery, cancer, use of oral contraceptives and hormone replacement therapy[7].

4- Procedure principles

The test is based on real-time PCR technology with TaqMan® probes. Each sample is analysed using:

- A pair of primers for the amplification of a fragment of the factor V gene (*F5*) where the G1691A mutation is located.
- A hydrolysis probe specific for the wild-type allele (1691G) labelled at the 5' end with HEX fluorophore and a hydrolysis probe specific for the mutant allele (1691A) labelled at the 5` end with FAM fluorophore. Both probes are labelled at the 3' end with a quencher that suppresses the fluorescence of the fluorophores when the probe is intact.

As the PCR reaction proceeds, the 5'→3'exonuclease activity of Taq polymerase cleaves the probes attached to their complementary sequence, separating fluorophore from quencher, and producing a fluorescent signal (in real time), which is proportional to the amount of PCR product generated and monitored in a real-time PCR instrument. Thus:

- In the case of homozygous wild-type (wt) samples for the analysed change (1691G/G), the HEX-labelled wt allele-specific probe binds to its complementary sequence of the amplified gene, and the following is observed:
    - fluorescent signal in the HEX channel (λmax 556 nm), and
    - no signal or weak signal in the FAM channel (λmax 518 nm).
- In the case of homozygous mutant (mut) samples for the analysed change (1691A/A), the FAM-labelled mut allele-specific probe binds to its amplified complementary sequence. In this case, the following is detected:
    - fluorescent signal in the FAM channel and
    - no signal or weak signal in the HEX channel.
- In the case of heterozygous samples for the analysed change (1691G/A), both probes shall bind to the amplified DNA sequences generating:
    - FAM channel signal and
    - signal in the HEX channel.

5- Kit contents

➔ GVS-FV-24 (24 tests)

- GVS-FV-PM: 1 vial x 96 µL Primer Mix (PM) - Blue cap
- GVS-FV-MM: 1 vial x 120 µL Master Mix (MM) - Red cap
- GVS-FV-C1: 1 vial x 15 µL Control WT (C1) - Green cap
- GVS-FV-C2: 1 vial x 15 µL Control MUT (C2) -Green cap with an orange sticker
- GVS-RB: 1 vial x 100 µL Reaction Blank (RB) - Natural cap

➔ GVS-FV-48 (48 tests)

- GVS-FV-PM: 2 vials x 96 µL Primer Mix (PM) - Blue cap
- GVS-FV-MM: 2 vials x 120 µL Master Mix (MM) - Red cap
- GVS-FV-C1: 1 vial x 15 µL Control WT (C1) - Green cap
- GVS-FV-C2: 1 vial x 15 µL Control MUT (C2) - Green cap with an orange sticker
- GVS-RB: 1 vial x 100 µL Reaction Blank (RB) - Natural cap

➔ GVS-FV-96 (96 tests)

- GVS-FV-PML: 1 vial x 460 µL Primer Mix (PML) - Blue cap
- GVS-FV-MML: 1 vial x 575 µL Master Mix (MML) - Red cap
- GVS-FV-C1L: 1 vial x 50 µL Control WT (C1L) - Green cap
- GVS-FV-C2L: 1 vial x 50 µL Control MUT (C2L) - Green cap with an orange sticker
- GVS-RB: 1 vial x 100 µL Reaction Blank (RB) - Natural cap

6- Kit storage

All the components of the kit must be stored between -30°C and -18°C upon receipt. Under these conditions, they are stable up to their expiry date.

Do not perform more than 3 freeze/thaw cycles on the kit vials as this may reduce the sensitivity of the assay and affect the results. If assays are to be performed with few samples, it is recommended to use aliquots of the reagents to reduce freeze/thaw cycles.

Due to the photosensitive nature of the Primer Mix, avoid continuous exposure to light.

7- Materials required but not supplied

General

- Disposable gloves
- Lab coat

Consumables

- Filter tips (P200, P20 and P10)
- 1.5 mL autoclaved tubes
- Compatible consumables for the real-time PCR instrument

Equipment

- Vortex mixer

- Centrifuge
- Micropipettes (P200, P20 and P10)
- Real-time PCR instrument, with FAM and HEX/VIC detection channels. The following devices have been validated:
    - StepOne™, 7500 and QuantStudio™ 6 Flex Real-Time PCR Systems, Applied Biosystems™
    - LightCycler® 96 System, Roche
    - DT Lite Real-Time PCR System, DNA-Technology
    - qTOWER³ G, Analytik Jena

8- Sample collection and preparation

Samples must be collected in line with the instructions for use of the collection device (not included with the kit) and under national and international guidelines.

The present test should only be performed with genomic DNA extracted from whole blood samples collected in EDTA anticoagulation agent or citrate. Heparin can interfere with the PCR process and should be avoided.

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 potentially infectious. When manipulating them, take the corresponding basic and universal precautions.

9- Usage procedures

➔ PCR setup

CAUTION!

- Define pre- and post-PCR work areas that should be kept separated to reduce the risk of contamination. Prepare the PCR in the pre-PCR area. Use a lab coat and disposable gloves.
- Work on ice or over a cool block. Minimize the time between preparation and the start of the assay.
- For each assay, it is recommended to test the contamination control (Reaction Blank) and both the Control WT (C1) and Control MUT (C2) included in the kit.

1. Thaw all the kit components before starting the assay. Vortex vigorously the Primer Mix vial and mix carefully the Master Mix vial. Centrifuge briefly to collect the volume at the bottom of the tubes.
2. Prepare the reaction mix for n+1 samples, using the quantities indicated in the following table:

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

Gently mix and centrifuge to ensure that all volume settles to the bottom of the tube.

3. Pipette 9 µL of this mix into the PCR plate/tubes.
4. Add into each well 1 µL of DNA, Reaction Blank, Control WT, or Control MUT.
5. Seal the plate/tubes using the appropriate sealer and centrifuge briefly to remove any bubbles. Ensure that all the volume settles to the bottom of the well.
6. Place the plate/tubes in the thermal cycler and set up the thermal cycler amplification program as described in the following section.

➔ Thermal cycler configuration

1. Set up the following readout channels:
   - FAM channel for detection of the mut probe.
   - HEX/VIC channel for detection of the wt probe.
2. Set up the following amplification program and start the run:

	Cycles	Temperature (°C)	Time (mm:ss)	Analysis
Denaturation	1	95	05:00	X
Cycles	50	95	00:15	X
		62	01:00	Single
Cooling	1	15	∞	X

➔ Disposal

Waste products shall be managed according to local regulations.

10- Results

➔ Results visualization

The analysis of the results is performed with the specific software of the real-time PCR instrument being used, and according to the manufacturer's instructions for use.

Once the run is finished, select the linear scale to visualize the amplification curves. It is recommended to check the correct behaviour of the obtained amplification curves:

- An amplification signal is considered positive if a quick and regular increase of fluorescence values (exponential) is observed (sigmoidal amplification) with Ct<35.
- A weak fluorescent, background signal or exponential signal with Ct>35 should not be considered a positive amplification. This assay allows the detection of alleles that differ by only one nucleotide and, therefore, in samples homozygous for one of the alleles, weak non-specific signals from the fluorophore used for the detection of the other allele can be observed. The appearance of these signals does not invalidate the assay.

A sample is considered positive if it produces an exponential amplification with Ct<35. A sample is considered negative if it produces a non-exponential amplification with low intensity or an exponential amplification with a Ct value >35.

CAUTION!

To determine the Ct value in each channel, adjust the threshold line as follows:

Select linear scale view and choose the region where the fluorescence signal is stable before exponential amplification. Place the threshold line above this background signal, so that it crosses close to the inflection point of the amplification curve. This line should slightly exceed the value of the highest fluorescence obtained with negative samples for the allele detected in this channel.

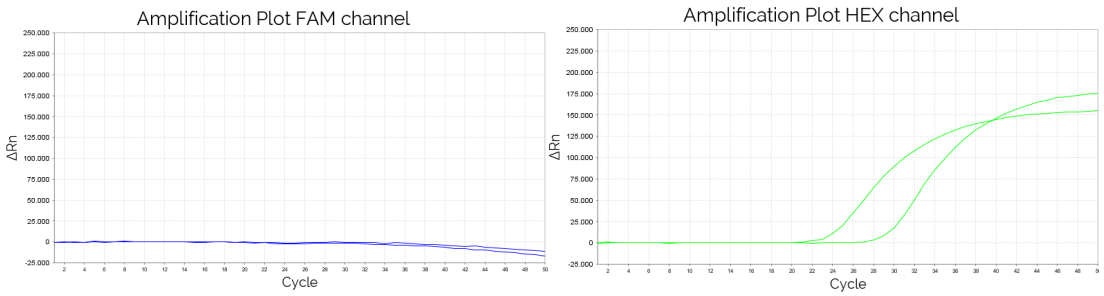
➔ Interpretation of results

The results obtained with this kit can be interpreted by visualization of the amplification curves in the FAM and HEX channels or by scatter plot of the end-point intensity values of the FAM and HEX channels.

Amplification curve

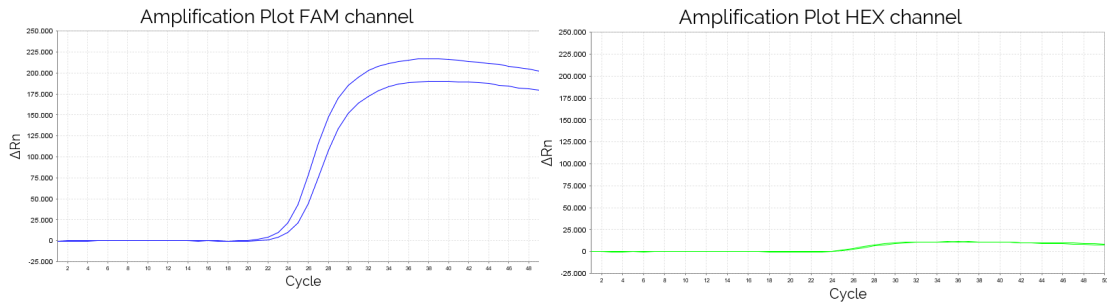
Select "linear scale" and observe the absence/presence of sigmoid amplification curves in each channel.

Homozygous wt sample (1691G/G)



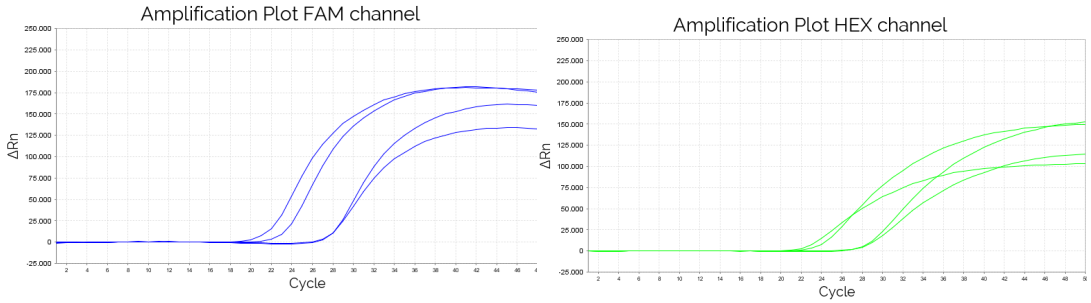
No signal or low intensity signal in FAM channel and exponential amplification in HEX channel (Ct<35)

Homozygous mut sample (1691A/A)



Exponential amplification in FAM channel (Ct <35) and no signal or low intensity signal in HEX channel

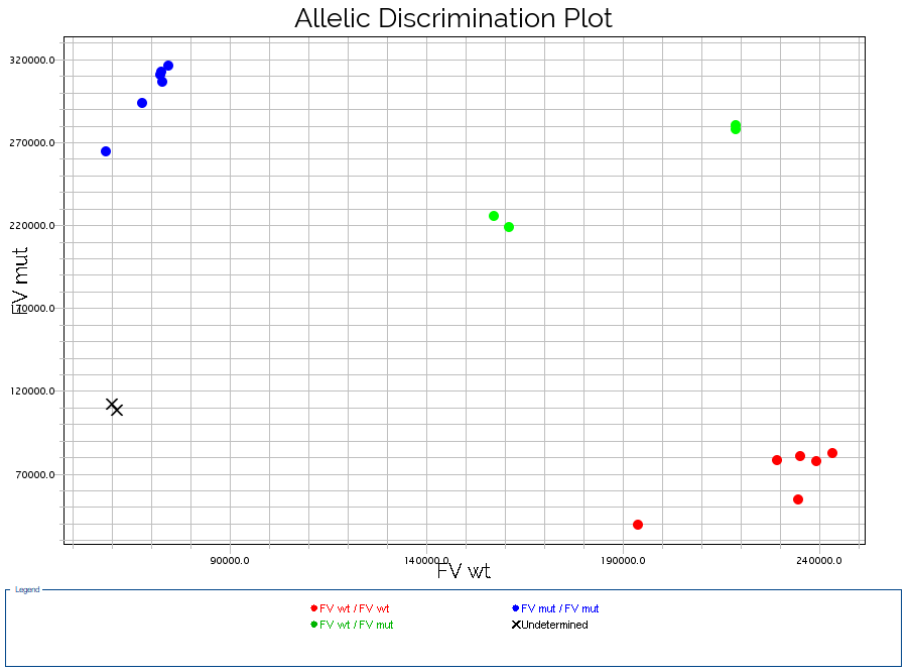
Heterozygous sample (1691G/A)



Exponential amplification signal (Ct<35) in both FAM and HEX channels

Scatter plot

Many real-time PCR software programs allow to automatically plot the fluorescence intensity data of one channel versus the other (allelic discrimination/genotyping). In this type of representation, data points located close to the X and Y axes correspond to homozygous genotypes for the allele detected with the fluorophore represented on the corresponding axis. Points located approximately in the middle of the axis correspond to heterozygous genotypes and the negative control (Reaction Blank) should appear at the bottom left, close to the origin.



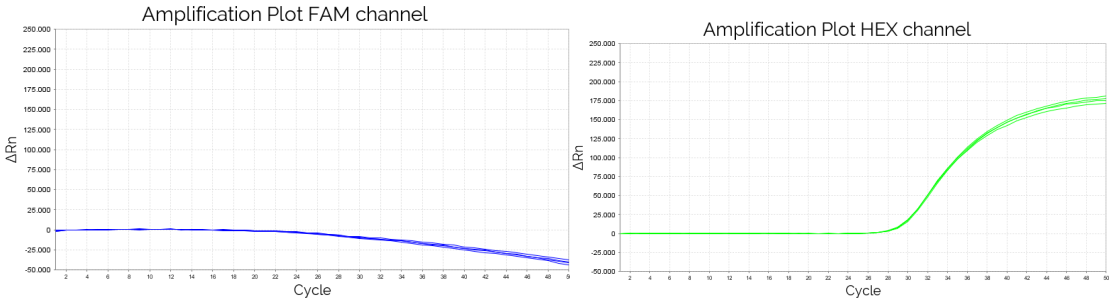
Plot showing heterozygous (green dots), homozygous mut (blue dots) and homozygous wt (red dots) samples for the G1691A mutation and a Reaction Blank, using Genvinset® Factor V G1691A kit.

11- Quality control

The kit includes a Reaction Blank, a Control WT (C1) and a Control MUT (C2) which must be tested in each assay. An adequate behaviour of these control samples is a guarantee of the proper performance of the reaction.

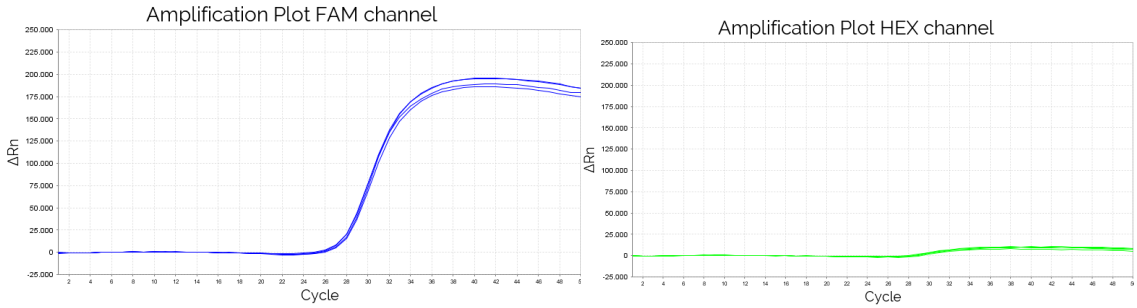
The results are considered valid if the following amplification pattern is obtained in the control samples:

Control WT (C1)



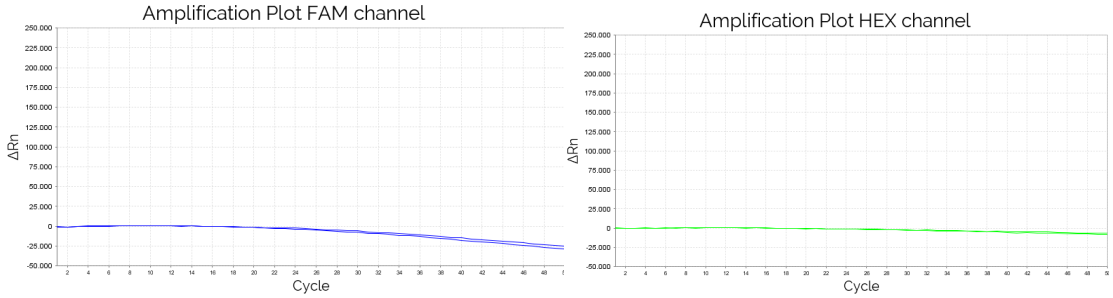
No signal or low intensity signal in FAM channel and sigmoidal signal in HEX channel (Ct<35)

Control MUT (C2)



Amplification signal in FAM channel (Ct<35) and no signal or low intensity signal in HEX channel

Reaction Blank



No signal in both FAM and HEX channels or amplification with Ct>35

The result is considered invalid and should be repeated if:

- An amplification curve with Ct<35 is observed in FAM and/or HEX channels in the Reaction Blank.
- Non-exponential amplification signal is observed or an amplification signal with Ct>35 appears in the Control WT or Control MUT wells.

If adequate behaviour is observed in the positive control reactions, proceed with the interpretation of the rest of the samples as indicated in the previous section.

12- Specific operation data

➔ Analytical specificity

Cross-reactivity has been measured in three independent Genvinset® Factor V G1691A kit validation studies that are described below.

In addition, primers and probes alignment has been checked *in silico*. The alignment of primer sequences is specific. Probes align specifically on the G1691A position (NCBI dbSNP rs6025; NM_000130.5:c.1601G>A) at the Factor V (*F5*) gene (OMIM: 612309). No cross-reaction phenomena with genomic DNA have been reported.

Interference has been studied by means of a bibliographic search. Heparin can inhibit Taq polymerase activity and compete with target nucleic acid, so the blood collected must be treated with other anticoagulants as stated in the "Sample collection and preparation" section. Some substances in blood are known to be PCR inhibitors: haemoglobin, hemin, bilirubin, bile salts, lactoferrin, and immunoglobulin G. The polymerase included in Genvinset® Factor V G1691A has demonstrated high resistance to inhibition, and Master Mix composition is designed to not be affected by interference substances. Nevertheless, potentially inhibitory substances must be eliminated during DNA extraction and purification protocol. Before delivering results with a diagnostic purpose, a validation assay with such extraction method should be done.

➔ Analytical sensitivity

An assay using 1:4 serial dilutions of three DNA samples, one wild-type (1691G/G) another one heterozygous (1691G/A) and a third homozygous mutant (1691A/A) for *F5* G1691A mutation was performed. DNA samples were extracted by a conventional automated extraction system (QIAsymphony, Qiagen), at concentrations of 92.1, 49.4 and 37.0 ng/µL respectively. The following data were obtained in terms of analytical sensitivity in the detection of wild-type and mutant alleles:

- Detection Limit of wild-type and mutant alleles = 0.20 ng/µL (Ct < 35)

➔ Diagnostic sensitivity and specificity

89 DNA samples obtained from several laboratories were analysed. They were previously genotyped by another commercial kit. The following results were obtained:

		Genvinset® Factor V G1691A		
	Genotype	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.

➔ Accuracy

Repeatability study consists in the measurement of within-run variability, through the analysis of replicas of every kind of sample that can be measured by the kit (homozygous and heterozygous samples). Each sample was analysed in duplicate.

Genvinset® Factor V G1691A showed a 100% repeatability.

A study to determine the reproducibility of the reagent was performed. It allowed to estimate the variability between runs, lots and operators. Samples used represent the full range of expected analyte that can be measured with Genvinset® Factor V G1691A, that is,

homozygous wild-type (G/G), homozygous mutant (A/A) and heterozygous (G/A). Three operators ran the assay in three different runs and tested three different reagent lots.

Genvinset® Factor V G1691A showed a 100% reproducibility.

➔ Trueness

The trueness of the analytical procedure of Genvinset® Factor V G1691A was assessed by comparison with a reference method. The study was developed as an internal validation of the reagent, in which trueness has been demonstrated with a 100% value. See the "Diagnostic Sensitivity and Specificity" section.

13- Procedure limitations

- The method detects the G/A SNP rs6025.
- Mutations or polymorphisms at annealing primer/probe sites are possible and may result in the lack of allele definition. Other technologies could be necessary to resolve the typing.
- All the recommendations mentioned in this document should be carefully followed. Any performances that do not meet such indications, can lead to poor results.
- 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 kit's performance.
- All Genvinset® reagent manipulations must be done according to good laboratory practice, being adapted to local regulations.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiry date. Dispose of expired reagents according to applicable regulations.
- The real-time PCR thermal cycler must be calibrated according to the manufacturer´s recommendations and should be used accordingly to the manufacturer's instructions.
- Data and result interpretation should be revised by qualified personnel.
- This product is an auxiliary tool for the diagnosis of patients with suspected thrombophilia. Use these results in conjunction with clinical data and results of other tests performed on the patient.

14- Troubleshooting guide

➔ No amplification signal is detected in any sample (neither in positive controls) or the intensity is very low

- The PCR instrument is not correctly programmed. The thermal profile is not correct/the reading channels are not correctly configured/the selected fluorophores are not suitable.
    - Check that the instrument has been correctly programmed.
- The positions of the samples and controls indicated on the worksheet do not coincide with the positions in which they have been placed in the device.
    - Correctly assign the position of the samples.
- The reagent does not work properly.

- Ensure that the kit is stored at an appropriate temperature (between -30°C and -18°C) and protected from light. Avoid unnecessary freeze/thaw cycles. Do not use it beyond the expiry date.

- The indicated amounts of each of the reagents have not been added to the reaction mix.
  - Check the volumes of each component added to the mix.
- The consumables used are not compatible with the equipment being used.
  - Make sure that you are using the correct consumables (compatible with the PCR instrument).

➔ No signal is detected in clinical samples (signal appears in positive controls)

- Poor quality of the DNA used.
  - Check the Absorbance ratio 260/280 and discard poor-quality samples. Avoid the presence of inhibitors (heparin, haemoglobin, hemin, bilirubin, bile salts, lactoferrin, immunoglobulin G). Repeat sampling and DNA extraction.
- Inadequate DNA concentration.
  - Check that the DNA concentration is above the limit of detection.
- Inhibition of amplification.
  - Collect whole blood in EDTA or citrate tubes.
- No sample was added.
  - Repeat assay making sure that samples have been added.

➔ Signal detected in the negative control

- Pipetting error.
  - Change the pipette tip each time DNA is added to a well. Check that the sample added to the well corresponds to what is written on the worksheet.
- Contamination of the Primer Mix/Master Mix/Reaction Blank vial.
  - Repeat the assay with fresh aliquots.
- The PCR preparation area is contaminated.
  - Clean surfaces, instruments, lab coats, and change consumables and reagents. Repeat the assay.

➔ Fluorescence intensity varies between samples or abnormal amplification curves are detected

- Dirtiness outside the reaction tube interferes with the detection of fluorescence.
  - Clean the equipment thoroughly. Check that the outside of the tubes/plate is clean. Handle the plates/tubes with gloves.
- The volume is not at the bottom of the well or there are bubbles.
  - Centrifuge the plates/tubes before inserting them into the thermal cycler.
  - Check if there are bubbles. If so, remove them.
- The plate/tubes have not been sealed properly.
  - Repeat the assay checking that the plates/tubes have been sealed correctly.
- DNAs with different concentrations have been used or the sample used contains a reaction inhibitor.
  - Repeat the sampling and DNA extraction.
- Presence of polymorphisms or mutations at the probe/primer binding sites.
  - Contact our Technical Support department through *customersupport@bdrdiagnostics.com*.

15- References

1) Lane, D. A. & Grant, P. J. Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. *Blood* **95**, 1517–1532 (2000).
2) Reitsma, P. H. & Rosendaal, F. R. Past and future of genetic research in thrombosis. *J Thromb Haemost* **5 Suppl 1**, 264–269 (2007).
3) Bertina, R. M. *et al.* Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* **369**, 64–67 (1994).
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5) Nicolaes, G. A. F. & Dahlbäck, B. Factor V and thrombotic disease: description of a janus-faced protein. *Arterioscler Thromb Vasc Biol* **22**, 530–538 (2002).
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7) Franchini, M. Utility of testing for factor V Leiden. *Blood Transfusion* **10**, 257 (2012).
8) Rosendaal, F. R. & Reitsma, P. H. Genetics of venous thrombosis. *J Thromb Haemost* **7 Suppl 1**, 301–304 (2009).
9) Rees, D. C. The population genetics of factor V Leiden (Arg506Gln). *Br J Haematol* **95**, 579–586 (1996).

16- Notice to purchaser

- This product has been developed for *in vitro* diagnostic purposes.
- When the kit is used within the territory of any Member State of the European Union, should there be any serious incident regarding the use of the kit, the user must report it to the manufacturer (*regulatory@bdrdiagnostics.com*) and the Competent Authority of their country and/or the patient´s country. Serious incident reporting for all other countries must be conducted according to local requirements for each country.
- Blackhills Diagnostic Resources, S.L.U. products should not be resold, modified for reselling or used to manufacture other commercial products without the written consent of Blackhills Diagnostic Resources, S.L.U.
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- FAM™ and HEX™ may be covered by one or more patents owned by Applied Biosystems, LLC. The purchase price of this product includes limited, nontransferable rights.
- TaqMan® is a registered trademark of Roche Molecular Systems, Inc.
- Genvinset® is a trademark of Blackhills Diagnostic Resources, S.L.U.

17- Changes control

Version	Description of the modification
Rev. 06	Modification of PCR annealing temperature, from 64°C to 62°C.

Rev. 07	Correction of typos and translation mistakes. New sections: Accuracy and Trueness. Information regarding intended user, intended patient and interferences has been added.
Rev. 08	Change in Control WT and Control MUT reference and volume. Change in the list of validated instruments. Insertion of UDI-DI codes.

18- Explanation of symbols used on the labels

IVD	*In vitro* diagnostic medical device		Expiration date
REF	Catalogue number		Contents sufficient for <n> tests
LOT	Lot number		Manufacturer
	Temperature limit		Keep away from sunlight
	Positive control		Consult electronic Instructions For Use document
CE	This product fulfils the requirements of Directive 98/79/EC on *in vitro* diagnostic medical device		