RIDA®GENE Factor V

REF PY1210

R-Biopharm AG, An der neuen Bergstrasse 17, 64297 Darmstadt, Germany
+49 (0) 61 51 81 02-0 / +49 (0) 61 51 81 02-20 / www.r-biopharm.com

1. Intended use

For *in vitro* diagnostic use. The RIDA®GENE Factor V kit uses real-time PCR to qualitatively detect a point mutation of G to A at position 1691 in the human factor V gene (Factor V Leiden mutation) in genomic DNA that was isolated from human EDTA whole blood samples.

The RIDA®GENE Factor V kit is intended to support the diagnosis in the assessment of patients with suspected thrombophilia.

2. Summary and explanation of the test

Thromboses are a major medical problem that increases with age. Roughly 1 in 100,000 people under 40 years develop venous thrombosis. This probability increases in people over 75 years to 1 in 100 people per year[1]. Factor V Leiden is considered the most common genetic factor that is described in combination with the development of venous thromboembolism[2]. In this case, it is a point mutation in the factor V gene at position 1691 that leads to a base exchange from G to A. As a result, the binding site for activated protein C with anticoagulant effect is no longer available (APC resistance), and factor V is broken down less compared with the wild type, which results in a hypercoagulable state[3]. Factor V Leiden is widely distributed with high prevalence in Europa and with practically no cases in Asia or Africa[2]. The probability of developing venous thrombosis is 3 to 8 times higher with a genetic mutation in the factor V gene if this mutation is heterozygous. For a homozygous mutation at position 1691, the probability increases 10 to 80 times. In addition, the chance of developing venous thrombosis at a young age also increases in this case[2]. If other factors, such as a mutation in the factor II gene (G20210A), are present with this mutation, the risk of recurrent thromboses increases as well[1].

3. Test principle

The RIDA®GENE Factor V kit uses real-time PCR to qualitatively detect a point mutation/single nucleotide polymorphism (SNP) of G to A at position 1691 in the human factor V gene (Factor V Leiden mutation) in genomic DNA that was isolated from human EDTA whole blood samples.
After DNA isolation, the specific gene fragment (wild type or mutation) is amplified. The amplified target sequences are detected using hydrolysis probes that are labeled with a quencher at one end and a fluorescent reporter dye (fluorophore) at the other. The probes hybridize to the amplicon in the presence of a target sequence. During extension, Taq-Polymerase separates the reporter from the quencher. The reporter emits a fluorescent signal that is detected by the optical unit of a real-time PCR instrument. The fluorescent signal increases with the quantity of formed amplicons.

4. Reagents provided

The reagents in the kit are sufficient for 100 determinations.

Table 1: Reagents provided

Kit code	Reagent	Amount		Lid color
1	Reaction Mix	2 ×	1050 µL	yellow, ready for use
2	Taq-Polymerase	1 ×	80 µL	red, ready for use
A	Control A	1 ×	200 µL	blue, ready for use
B	Control B	1 ×	200 µL	green, ready for use

5. Storage instructions

- Please follow the handling guidelines in Table 2 and store the kit directly after use according to the information specified.
- All reagents must be stored away from light at -20 °C and, if unopened, can be used until the expiration date printed on the label. After the expiration date, the quality guarantee is no longer valid.
- All reagents should be carefully thawed prior to use (e.g., in a refrigerator at 2 °C - 8 °C).
- Repeated freezing/thawing of up to 20 times does not have an impact on the test property (if necessary, create aliquots after the first thaw and refreeze reagents immediately).
- Cool all reagents appropriately during PCR preparation (2 °C - 8 °C).

Table 2: Storage conditions and information

	Storage temperature	Maximum storage time
unopened	-20 °C	Can be used until the printed expiration date
opened	-20 °C	20 freeze-thaw cycles

6. Reagents required but not provided

6.1 Laboratory equipment

The following equipment is needed for using the RIDA®GENE Factor V kit:

Equipment
Extraction platform: Maxwell® RSC (Promega)
Real-time PCR instruments: • LightCycler® 480 II (Roche) • Cobas z 480 Analyzer (Roche) • Mx3005P (Agilent Technologies) • ABI 7500 (Applied Biosystems) • CFX96™ (Bio-Rad)
RIDA®GENE Color Compensation Kit IV (PG0004) (R-Biopharm) when using LightCycler® 480 II and cobas z 480 Analyzer
Real-time PCR consumables (plates (low profile, white wells, clear frame), reaction vials, films)
Centrifuge with rotor for plates
Vortexer
Pipettes (0.5 - 20 µL, 20 - 200 µL, 100 - 1000 µL)
Pipette tips with filters
Powder-free disposable gloves

For questions on the use of equipment for automated processing, please contact R-Biopharm AG at pcr@r-biopharm.de.

7. Warnings and precautions for the users

For *in vitro* diagnostic use only.
This test must be carried out only by qualified laboratory personnel. The guidelines for working in medical laboratories must be followed.
Always adhere strictly to the operating manual when carrying out this test.
Do not pipette samples or reagents using your mouth. Avoid contact with broken skin and mucous membranes.
Wear personal protective equipment (appropriate gloves, lab coat, safety glasses) when handling reagents and samples, and wash hands after completing the test.
Do not smoke, eat, or drink in areas where samples are handled.
Separate rooms, special clothing, and instruments for extraction, PCR preparation, and PCR must be used to prevent cross-contamination and false-positive results.
Avoid contaminating the samples and components of the kit with microbes and nucleases (DNase/RNase).
Clinical samples must be viewed as potentially infectious and must be disposed of appropriately, like all reagents and materials that come into contact with potentially infectious samples.
Do not exchange or mix the components (Reaction Mix, Taq-Polymerase, Control A, Control B) of one kit lot with the components of another lot.
Do not use the kit after the expiration date. Users are responsible for the proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

Further details on the Safety Data Sheet (SDS) can be found under the item number at https://clinical.r-biopharm.com/search/.

For users in the European Union: Report all serious adverse events associated with the product to R-Biopharm AG and the appropriate national authorities.

8. Collection and storage of samples

8.1 Sample storage

This test was developed for the examination of human EDTA whole blood samples. Store the samples at room temperature for up to 24 hours and at 2 °C - 8 °C for up to 72 hours until DNA extraction[(4)]. Microbial contamination of the samples must be avoided. The use of heat-inactivated, lipemic, hemolytic, icteric, or cloudy samples can lead to false results.

8.2 Preparation of samples

8.2.1 DNA isolation from EDTA whole blood

A commercially available DNA isolation kit or DNA extraction system (e.g., Maxwell® RSC Instrument (Promega)) is recommended for the isolation of DNA from EDTA whole blood. The manufacturer's instructions must be observed.
When using the Maxwell® RSC Instrument (Promega), it is recommended to mix the blood samples for at least 5 minutes at room temperature. Add 30 µL proteinase K to a 2-mL reaction vial for sample preparation. Add 200 µL from the blood sample and 300 µL from the lysis buffer. Vortex the solution for 10 seconds and incubate at 56 °C for 20 minutes. Use 100 µL of the elution buffer for the extraction. The manufacturer's further instructions must be observed.

9. Test procedure

9.1 Preparation of the Master Mix

The total number of the reactions needed for PCR (samples and control reactions) must be calculated. Control A and Control B must be run during every test run.

Adding an additional 10 % volume to the Master Mix is recommended in order to compensate for the pipetting loss (see Table 3). Before use, thaw the Reaction Mix, Taq-Polymerase, Control A, and Control B, vortex (except for Taq-Polymerase), and centrifuge for a short time. Reagents must always be cooled appropriately during the work steps (2 °C - 8 °C).

Table 3: Example for the calculation and production of the Master Mix for 10 reactions

Kit code	Components of the Master Mix	Quantity per reaction	10 reactions (plus 10 %)
1	Reaction Mix	19.3 µL	212.3 µL
2	Taq-Polymerase	0.7 µL	7.7 µL
	Total	**20 µL**	**220 µL**

Mix the Master Mix and then centrifuge for short time.

9.2 Preparation of the PCR mix

Pipette 20 µL of the Master Mix into each reaction vial (plates).

Samples: Add 5 µL eluate to each pre-pipetted Master Mix.

Controls: Add 5 µL of Control A to each pre-pipetted Master Mix.

Add 5 µL of Control B to each pre-pipetted Master Mix.

Seal the plates, briefly centrifuge at slow speed, and transfer to the real-time PCR instrument. Start PCR according to PCR instrument set-up (Table 4, Table 5).

9.3 PCR instrument set-up

9.3.1 Universal real-time PCR profile

To harmonize the RIDA®GENE assays, the RIDA®GENE Factor V kit was verified in the universal profile. This makes it possible to combine DNA and RNA assays with each other. Reverse transcription therefore comes first in the universal profile.

Table 4: Universal real-time PCR profile for LightCycler® 480 II and cobas z 480 Analyzer

Reverse transcription		10 min, 58 °C
Initial denaturation		1 min, 95 °C
Cycles		45 cycles
PCR	Denaturation	10 sec, 95 °C
	Annealing/extension	15 sec, 60 °C
Temperature transition rate/ ramp rate		Maximum

Note: **Annealing and extension take place in the same step.**

Table 5: Universal real-time PCR profile for Mx3005P, ABI 7500, and CFX96™

Reverse transcription	10 min, 58 °C
Initial denaturation	1 min, 95 °C
Cycles	45 cycles
PCR Denaturation Annealing/extension	15 sec, 95 °C 30 sec, 60 °C
Temperature transition rate/ ramp rate	Maximum

Note: Annealing and extension take place in the same step.

9.4 Detection channel setting

Table 6: Selection of appropriate detection channels

Real-time PCR instrument	Detection	Detection channel	Comment
Roche LightCycler® 480 II	Factor V WT	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required.**
	Mutation	533/580	
Roche cobas z 480 Analyzer	Factor V WT	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required.**
	Mutation	540/580	
Agilent Technologies Mx3005P	Factor V WT	FAM	-
	Mutation	HEX	
Applied Biosystems ABI 7500	Factor V WT	FAM	**Set the ROX passive reference dye to none.**
	Mutation	VIC	
Bio-Rad CFX96™	Factor V WT	FAM	-
	Mutation	HEX	

10. Quality control - indication of instability or expiration of reagents

Samples are evaluated using the analysis software of the real-time PCR instrument according to the manufacturer's instructions. Control A and Control B must show the correct results (see Table 7).

Table 7: A valid PCR run must meet the following conditions:

Sample	FAM	VIC	Target gene Ct
Control A	+	-	See Certificate of Analysis
Control B	-	+	See Certificate of Analysis

* + = positive
  - = negative

If the two controls, Control A and Control B, do not comply with the specifications, repeat the entire PCR run. If the specified values are not met, check the following items before repeating the test:

- Expiration date of the reagents used
- Functionality of the equipment being used
- Correct test procedure

If the conditions are still not fulfilled after repeating the test, please consult the manufacturer or your local R-Biopharm distributor.

11. Result interpretation

The sample results are interpreted according to Table 8.

Table 8: Result interpretation*

Detection of		
Factor V WT	***Mutants***	**Result**
+	**-**	**Factor V wild type (homozygous) detectable**
-	**+**	**Factor V mutation in position 1691 (homozygous) detectable**
-	**-**	**Target sequence not detectable, invalid**
+	**+**	**Heterozygous phenotype**

* + = positive
- = negative

The PCR run cannot be evaluated if the controls in the detection system do not show amplifications or none of the target channels show a positive result. The whole PCR run must be repeated.

The PCR run cannot be evaluated if the sample shows no amplification in the detection system. In this case, either the DNA was not added or unsuitable template DNA (quality, PCR inhibitors) was used. The extracted sample should be re-amplified or the isolation and purification of the sample should be improved.

12. Limitations of the method

1. The RIDA®GENE Factor V kit detects position 1691 and any single nucleotide polymorphism (SNP) in the human factor V gene (Factor V Leiden mutation) from human EDTA whole blood samples. A connection between the level of the determined Ct value and the occurrence of severe clinical symptoms cannot be derived from this. The results obtained must always be interpreted in combination with the complete clinical symptoms.
2. The diagnosis should not be based on the result of the molecular biological analysis alone, but should always take the patient's medical history and symptoms into account.
3. This test is only valid for EDTA whole blood samples.
4. Improper specimen extraction, transport, storage, and handling can produce false-negative results.
5. The presence of PCR inhibitors can lead to false-negative or invalid results.
6. This assay should be performed in compliance with the regulation on good laboratory practice (GLP). Users must follow exactly the manufacturer's instructions when performing the test.
7. The German Genetic Diagnostics Act (GenDG) requires a thorough explanation and written consent of the patients for all genetic analyses in accordance with the GenDG.

13. Performance characteristics

13.1 Analytical performance characteristics

13.1.1 Analytical specificity

Interfering substances

The presence of PCR inhibitors and interfering substances can lead to invalid results. Therefore, the effects of various substances that could be present in the corresponding samples due to their widespread occurrence were investigated. Substances that could potentially significantly influence the test results were examined initially at high concentrations (simulation of the worst case) in an interference screen. If a potential interference was found in this interference screen for an examined substance, a dose-effect relationship was established between the concentration of the substance in question and the interference.
No interferences were found for the substances listed in Table 9.

Table 9: Potentially interfering substances

Potentially interfering substance	Concentration
Medunasal Heparin 500 IU vials (Heparin)	15 U/mL
Cholesterol	3 mg/mL
Bilirubin	0.1 mg/mL
Hemoglobin	0.2 mg/mL
K_2EDTA	1.8 mg/mL

13.1.2 Precision

The precision of the Factor V kit was determined for the following levels of consideration.

Intra-assay precision: Determination of 5 control samples using 20 replicates each on LightCycler® 480 II under identical conditions.

Inter-assay precision: Determination of 5 control samples in 20 runs in duplicate on 10 work days (2 runs per day) performed by two different technicians under reproducible conditions.

Testing for *intra*- and *inter*-assay precision was carried out using three different lots.

The precision data were obtained using five control samples, as well as the controls belonging to the assay.

The maximum obtained coefficient of variation of the measurements with the RIDA®GENE Factor V kit on LightCycler® 480 II was 1.37 %.

Table 10: Results of the precision of the RIDA®GENE Factor V kit for 1691G.

Ct mean value/CV		Intra-assay			Inter-assay			Inter-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	27.9	28.3	28.3	28.3	28.2	28.4	28.3
	CV (%)	0.45 %	0.37 %	0.36 %	0.95 %	1.05 %	1.04 %	1.03 %
2	Ct	23.3	23.6	23.6	23.5	23.4	23.6	23.5
	CV (%)	0.38 %	0.28 %	0.19 %	1.07 %	1.37 %	1.25 %	1.24 %
3	Ct	22.0	22.3	22.3	22.3	22.3	22.4	22.3
	CV (%)	0.49 %	0.37 %	0.43 %	1.08 %	1.13 %	1.18 %	1.14 %
4	Ct	20.8	21.0	21.0	21.0	21.0	21.1	21.1
	CV (%)	0.43 %	0.44 %	0.51 %	1.19 %	1.26 %	1.31 %	1.27 %
5	Ct	neg.	neg.	neg.	neg.	neg.	neg.	neg.
	CV (%)	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 11: Results of the precision of the RIDA®GENE Factor V kit for 1691A.

Ct mean value/CV		Intra-assay			Inter-assay			Inter-lot
		Kit lot 1	Kit lot 2	Kit lot 3	Kit lot 1	Kit lot 2	Kit lot 3	Kit lots 1-3
1	Ct	neg.	neg.	neg.	neg.	neg.	neg.	neg.
	CV (%)	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2	Ct	24.2	24.2	24.2	24.2	24.5	24.6	24.4
	CV (%)	0.53 %	0.65 %	0.73 %	1.01 %	0.86 %	0.73 %	1.21 %
3	Ct	23.1	23.1	23.2	23.0	23.4	23.5	23.3
	CV (%)	0.67 %	0.58 %	0.75 %	0.76 %	0.98 %	1.02 %	1.35 %
4	Ct	21.8	21.8	21.8	21.7	22.0	22.1	21.9
	CV (%)	0.73 %	0.63 %	0.65 %	0.76 %	0.77 %	0.88 %	1.21 %
5	Ct	28.8	28.9	28.8	28.7	29.1	29.1	29.0
	CV (%)	0.39 %	0.72 %	0.41 %	0.56 %	0.77 %	0.92 %	1.04 %

14. Version history

Version number	Section and designation
2022-03-08	Release version

15. Explanation of symbols

General symbols

IVD	For in vitro diagnostic use
	Observe operating manual
LOT	Batch number
	Use before
	Storage temperature
REF	Item number
	Number of tests
	Date of manufacture
	Manufacturer

Test-specific symbols

Reaction Mix	Reaction Mix
Taq-Polymerase	Taq Polymerase
Control A	Control A
Control B	Control B

16. References

1. Zhang S, Taylor AK, Huang X, Luo B, Spector EB, Fang P, et al. Venous thromboembolism laboratory testing (factor V Leiden and factor II c.*97G>A), 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2018;20(12):1489-98.
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3. Safavi-Abbasi S, Di Rocco F, Nakaji P, Feigl GC, Gharabaghi A, Samii M, et al. Thrombophilia Due to Factor V and Factor II Mutations and Formation of a Dural Arteriovenous Fistula: Case Report and Review of a Rare Entity. Skull Base. 2008;18(2):135-43.
4. Probenlagerung vor Extraktion: Clinical and Laboratory Standards Institute (CLSI). Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline. CLSI document MM13-A, Vol. 24, N. 31, 2005