



Factor V Leiden (FV)

REF: RT-50

Determination of the G1691A mutation on Factor V Leiden in Real Time PCR

INTRODUCTION AND PURPOSE OF USE

The Fattore V di Leiden (FV) Kit is a qualitative test that allows the allelic discrimination, by means of *Real Time PCR*, of Leiden mutation (G1691A) associated to Hereditary Thrombophilia.

The Procedure allows the amplification of Wild-type alleles and mutated alleles of Factor V Leiden. Allelic discrimination is performed making a scatter plot of mutated allele's fluorescence (Y axis) versus wild-type allele's fluorescence (X axis), discriminating in this way the three possible genotype: Homozygote Wild-Type, Homozygote Mutated and Heterozygote Mutated.

The analysis of the results is made by an instrument of Real Time PCR, composed by a thermal cycler with a system of fluorescence detection.

CONTENT

The kit contains reagents enough to perform 48 amplification tests

	Quantity	Description
R1	3 x 210 µl	Amplification mMix dNTPs, Tris-HCl, KCl, MgCl ₂ , Taq Polymerase, Nuclease-free water
R2	3 x 130 µl	Factor V probes Mix Upstream primer 1, Upstream primer 2, downstream primer, Target probes (FAM for Wild Type and VIC for Mutant), Nuclease-free water
R3	3 x 10 µl	Positive Control Homozygote Wild-Type cloned DNA corresponding to FV Wild-Type gene.
R4	3 x 10 µl	Positive Control Homozygote Mutated cloned DNA corresponding to FV Mutated gene.
R5	3 x 10 µl	Positive Control Heterozygote Mutated cloned DNA corresponding to FV Wild-Type gene and Mutated.
R6	1 x 20 µl	Negative Control

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge (12,000 - 14,000 rpm);
Micropipettes and Sterile tips with aerosol filter;
Vortex;
Plastic materials (microplate and optica adesive cover);
Real Time PCR Instrumentation.

ACCESSORY PRODUCTS

EX05 – DNA Extraction from Blood, Uretral, Vaginal and Cervical Swab

The kit allows the DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 50 samples.

SAMPLES AND STORAGE

The Factor V Leiden (FV) system must be used with extracted DNA from the following biological samples: Blood EDTA and buccal swab. Collected samples must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.

Store the sample at -20°C if it is used after 3 days.

PRECAUTIONS USE

This kit is for *in vitro* diagnostic (IVD), for professional use only and not for *in vivo* use.

After reconstitution of the amplification master mix, it must be used in once (16 reactions). Do not re-frost already used material.

At all times follow Good Laboratory Practice (GLP) guidelines.

Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.

Avoid any contact between hands and eyes or nose during specimens collection and testing.

Handle and dispose all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.

Keep separated the extraction and the reagents preparation. Never pipette solutions by mouth.

Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.

Wash hands carefully after handling samples and reagents. Do not mix reagents from different lots.

It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).

Do not eat, drink or smoke in the area where specimens and kit reagents are handled.

Read carefully the instructions notice before using this test. Do not use beyond the expiration date which appears on the package label.

Do not use a test from a damaged protective wrapper.

LIMIT OF THE METHOD

The extreme sensitivity of gene amplification may cause false positives due to cross-contamination between samples and controls. Therefore, you should:

- physically separate all the products and reagents used for amplification reactions from those used for other reactions, as well as from post-amplification products;
- use tips with filters to prevent cross-contamination between samples;
- use disposable gloves and often change them;
- carefully open test tubes to prevent aerosol formation;
- close every test tube before opening another one.

The proper functioning of the amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.

As with any diagnostic device, the results obtained with this product must be interpreted in consideration of all the clinical data and other laboratory tests done on the patient.

As with any diagnostic device, there is a residual risk of obtaining invalid results, false positives and false negatives with this product.

STORAGE AND STABILITY

Store the product Factor V Leiden (FV) at -20°C.

An intact and well stored product has a stability of 12 months from the date of production.

Do not use beyond the expiration date which appears on the package label.

ANALYTICAL PROCEDURE

ABI 7500 INSTRUMENT SOFTWARE SETTING:

Turn the instrument and the computer on and open the control software. Click on "**Advance Setup**": by default the software will show the page "**experiment properties**". Write in the "**experiment name**" the file name, choose the type of instrument (**ABI7500** o **ABI7500fast**), the type of reaction (**Genotyping**), the type of used reagent (**Taqman®Reagents**) and the reaction time of analysis (**Standard = 2 hours to complete a run**).

Open the page named "**page setup**".

In the window "**Assign SNP assay to the selected wells**" open "**Create new SNP Assay**" and set:

SNP Assay Name: FV:

	Reporter	Quencer
Allele 1 Name: FV WT	FAM	NFQ
Allele 2 Name: FV MUT	VIC	NFQ

In the page "**plate setup**", move on the area "**Assign Sample to the selected Wells**": set the name of the analyzing samples, of positive controls and negative controls.

Choose an area of the plate where positive controls will be placed: select in the blank "**Assign SNP assay to the selected well**" and assign the FV SNP Assay. After set these tasks:

- “task Positive control Allele1/Allele1” for FV Wild Type homozygote target;
- “task Positive control Allele2/Allele2” for FV mutated homozygote target;
- “task Positive control Allele1/Allele2” for FV mutated heterozygote target;

Choose an area of the plate where negative control will be placed: select "**Assign SNP assay to the selected well**" the "task Negative control" for FV SNP.

Select an area of the plate where samples will be placed: select the wells and set FV SNP. Link every well to a sample, through the window "**Assign samples to selected wells**".

For each sample, select in the blank "**Assign SNP to selected wells**" the "**task UnKnown (U)**" for the FV SNP.

WT	C1	C9					
MUT	C2	C10					
ET	C3	C11					
	C4						
	C5						
	C6						
	C7						
N	C8						

Set ROX as passive reference, using as normalizer of detecting fluorescence.

Open "**Run Method**" (sheet **Graphic View**) and set the right thermal cycling:

cycles	Pre PCR Read	denaturation	Annealing extension	Post PCR Read
1	60°C 1min			
1		95°C 10min		
35		95°C 15sec	60°C 1min	
1				60°C 1min

In the window "**Reaction volume plate per well**" set a volume of 25 µl.

After making the plate, and correctly inserting it in the instrument, press the button "**Start Run**".

PREPARATION OF THE REACTIONS:

Defrost a tube of **Amplification mMix**;
Defrost a tube of **Factor V probes Mix**;
Mix carefully by vortex **200 µl of Amplification mMix** and **120 µl of Factor V probes Mix** (the mix as produced is enough to prepare **16 reactions** of amplification: **3 positive controls, 1 negative control and 12 samples**).

Distribute, in the amplification plate, **20 µl of just reconstituted mix** in chosen positions and already setted on the instrument software.

Distribute, in the negative control position, **5 µl** of solution taken by the **negative control** vial.

Distribute, in chosen position for each sample, **5 µl** of **corresponding sample**.

Distribute, in chosen positions for the positive controls, **5 µl** of **Positive Control Homozygote Wild-Type, Positive Control Homozygote Mutated and Positive Control Heterozygote Mutated**.

Seal up accurately the plate using an optichal adesive film and verify that there aren't air bubbles in the mix, to avoid the amplification interferences.

Transfer the plate in the instrument and push the button "**Start Run**".

INTERPRETATION OF RESULTS

The use of positive and negative control in each amplification session allows to verify the correct functioning of the amplification mix and the absence of any contamination.

A regular functioning of the amplification mix can be verified analyzing the correct position of positive controls and negative controls on the scatter plot.

- Positive control Homozygote Wild-Type: horizontal position on X axis (down on the right) Ct < 25
- positive Control homozygote Mutated: vertical position on Y axis (up on the left) Ct < 25

- positive Control heterozygote Mutated: diagonal position between two axis $Ct_{wt} e_{mut} < 25$
- negative Control: placed at the origin of cartesian plane (down on the left)

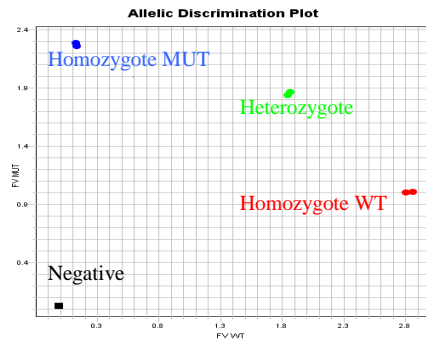
Genotyping tests (allelic discrimination) are endpoint experiments: fluorescence data are collected at the end of the reaction (Post PCR Read) and subtracted to initial read fluorescence (Pre PCR Read).

The software makes a scatter plot with obtained results: Y axis is the normalized fluorescence of Mutated Allele, while X axis shows the normalized fluorescence of Wild-Type Allele. The diagnosis obtained with the comparison between unknown samples and Homozygote Wild-Type, Homozygote Mutated and Heterozygote Mutated, given by the kit. Selecting the controls, we obtain their disposition on the scatter plot, depending of their relation between fluorescence emitted by two probes FAM (Wild-Type) and VIC (Mutated).

ANALYSIS OF RESULTS

At the end of the amplification reaction, the software automatically shows the obtained results in the "Allelic Discrimination Plot".

The ABI7500 Fast instrument automatically performs the genotyping of unknown samples by comparing controls versus homozygous wild-type, heterozygous mutated and homozygous mutated contained in the kit. It is recommended to verify correct placement of each individual sample on the scatter plot. For viewing the report containing all data obtained during the analysis, click the sheet "view table well".



The position of the negative control in other plot region could be a contamination of the reaction mix.

PERFORMANCES

Clinical sensitivity:

For the purposes of this evaluation IT is considered as clinical sensitivity the skill of determining true heterozygote and homozygote mutated samples in the totality of screened samples. The test is performed following the method advices. Positive samples are confirmed with another CE disposable method.

Samples	Heterozygote		Homozygote Mutated	
	Pos	Neg	Pos	Neg
Donors EDTA blood	19	0	6	0

Clinical sensitivity is 100% for material extracted from EDTA blood.

Diagnostic Specificity:

For the purposes of this evaluation is considered as diagnostic specificity the skill of the method of determining real negative samples. The diagnostic specificity of the system is valued analyzing human genomic samples tested and confirmed as negative with another disposable system.

Samples	N	Positive	Negative
Donors EDTA blood	50	0	50

Diagnostic specificity is 100% for material extracted from EDTA blood.

Analytical Specificity:

Test's specificity is guaranteed by the use of specific primers for determining Factor V gene and of probes intentionally designed on G1691A mutation.

The alignment of the choose regions for specific primers' hybridization with available sequences of present in database, demonstrated: their conservation and the complete specificity for the analyzed targets. Samples that are defined as positive for a determined genotype as much must be recognized by the amplification system.

INTERFERENCES:

Verify that in DNA extracted from the sample there aren't nucleoproteins and haemoglobin, in way of exclude possible inhibition of PCR reactions. The interference due to contaminants can be highlighted through the spectrophotometric analysis and obtained data report at 260 nm (maximum absorption of Nucleic Acids) and 280 nm (maximum absorption of Proteins). A pure DNA might have a rate of approximately 1.8.

QUALITY CONTROL

It is therefore recommended to insert as quality control of every extraction session, amplification and detection of a negative sample and of a positive sample which have already tested before or referential material with known concentration.

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TROUBLESHOOTING

Presence, in the negative control, of the specific fluorescence for examining Target:

Possible causes	Solutions
Error displacing the positive sample or the positive control.	<ul style="list-style-type: none"> • Use only micropipettes and Sterile tips with aerosol filter • Always change tip between a sample and another • Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use • During analytical stage, distribute positive control for last • During analytical stage, before distribute negative control
Contamination of the amplification mixes	<ul style="list-style-type: none"> ○ Don't dispense the amplification mix with the same pipettes used in the extraction or detection area ○ Use only micropipettes and Sterile tips with aerosol filter ○ Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use ○ Close the amplification mix and return it at -20°C before open the other sample tubes
Badly sealed microplate	<ul style="list-style-type: none"> • Seal very carefully the microplate
Mistake during the instrument setup of the plate	<ul style="list-style-type: none"> • Check the correct correspondence between setting on the instrument and the real positions on the microplate (for samples, negative controls and standard)
Contamination of the preparation area for amplification reaction	<ul style="list-style-type: none"> • Clean surfaces and instruments with aqueous detergents that can degrade DNA, wash lab coats, replace test tubes and tips in use

Failure or incorrect amplification of positive control:

Possible causes	Solutions
Possible mistakes in the dispensation of the positive control	<ul style="list-style-type: none"> • Dispense carefully the positive control • Check the calibration of micropipettes used • Verify that the amount of positive control used corresponds to the method described in the Analytical Procedure (Sul)
Positive Control Degradation	<ul style="list-style-type: none"> • Test the mixture through a new batch of positive control
Wrong heat protocol	<ul style="list-style-type: none"> • Check the thermal cycler setting
The reaction mix is maintained at room temperature for too long	<ul style="list-style-type: none"> • Keep the amplification mix on ice during the use and store it at -20 ° C immediately after finishing the preparation of test samples

Failure or incorrect amplification of internal control in testing samples:

Possible causes	Solutions
Presence of possible interferences	<ul style="list-style-type: none"> • Carefully repeat sample extraction stage
Insufficient DNA extracted quantity	<ul style="list-style-type: none"> • Carefully repeat sample extraction stage • Accurately samples storage

	In vitro diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
	Lot (xxxx)
	Code
	Manufacturer
	Contains sufficient for <n> tests

EDMA code: 1601010300
CND: W0106010103



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