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FV Leiden G1691A SNP-Screen

Real Time Amplification Kit

NAME

FV Leiden G1691A SNP-Screen

INTRODUCTION

Cardiovascular diseases are lifethreatening conditions which affect up to 10% of the human population. Thrombotic complications, such as an acute myocardial infarction, ischemic stroke, pulmonary embolism, deep venous thrombosis are the major causes of morbidity and mortality in the world. Moreover, an increased tendency to develop thrombosis, called also “thrombophilia”, underlies the significant proportion of cases in the most common obstetric complications (recurrent pregnancy loss, fetal growth retardation, preeclampsia, abruptio placentae).

Recent data suggest a significant role of genetic background in predisposition toward thrombosis. Three important inherited thrombophilias which are responsible of the majority of thromboembolic events were discovered in patients with otherwise no apparent risk for thrombosis. Resistance to activated protein C caused by a guanine 1691 adenine (G1691A) mutation in factor V (factor V Leiden) was linked with an increased risk for venous thromboembolism. Heterozygosity for the factor V (FV) Leiden mutation is found in about 5% of the population and the mutation is responsible of 20–30% of venous thromboembolism events. A recently described guanine 20210 adenine mutation in prothrombin is associated with higher plasma prothrombin concentrations and it increased risk for venous thromboembolism and cerebral-vein thrombosis. Homozygosity for the cytosine 677 thymine (C677T) mutation in methylenetetrahydrofolate reductase (MTHFR) results in decreased synthesis of 5-methyltetrahydrofolate, the primary methyl donor in the conversion of homocysteine to methionine and the resulting increase in plasma homocysteine concentrations is a risk factor for thrombosis. The mutation responsible for this form is a C (cytosine) to T (thymine) substitution at nucleotide 677 of the coding sequence which converts an alanine to a valine residue. The mutation is responsible for reduced MTHFR activity, it is the most frequent cause of mild hyperhomocysteinemia and can be found in 5–15% of the population.

Identification of thrombophilia state in an individual could be helpful for the management of thrombosis-related and other complex human pathologies. It could also allow the physician to prescribe an appropriate prophylaxis for affected persons, aimed to prevent thrombotic complications in risk situations.

INTENDED USE

Kit **FV Leiden G1691A SNP-Screen** is a Real Time PCR test for detection of mutation in factor V (factor V Leiden).

PRINCIPLE OF ASSAY

The test is performed by the detection of the genetic variants within the FV gene using competitive allele-specific amplification in Real Time PCR. The kit contains 2 allele-specific primers in one tube: the 1st primers detect the “normal” wild type genome and they are labeled with FAM (channel Fam) while the 2nd primers detect the mutant genome and are labeled with R6G (channel R6G). The presence of positive fluorescence signal in one channel testifies the homozygote condition of genome and the positive fluorescence signals in both channels corresponds to the presence of the FV Leiden mutation in heterozygous form. The absence of a detectable signal is indicative for an inefficient DNA extraction or a PCR inhibition.

MATERIALS PROVIDED

- PCR Master Mix, 0,525 ml;
- Diluent, 0,525 ml;
- Taq Polymerase, 0,025 ml;
- Pos1 Control, DNA “FV(–/–)” (normal genotype FV G/G), 50 µl
- Pos2 Control, DNA “FV (+/–)” (heterozygous mutation FV G/A), 50 µl
- Pos3 Control DNA “FV(+/+)” (homozygous mutation FV A/A), 50 µl
- Neg Control C-, 200 µl

Contains reagents for 50 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

- Real Time Thermalcycler
- Reaction tubes
- Workstation
- Pipettors (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator
- Tube racks
- Disposable powder free gloves

Reagents non provided

- DNA isolation kit. The following isolation kits are recommended:
 - ⇒ **Genomic column DNA Express** – spin column extraction kit (Sacace, [REF](#) K-1-1/E)
 - ⇒ **DNA-Sorb-B** (Sacace, [REF](#) K-1-1/B)
 - ⇒ **QIAamp DNA Blood mini kit** (Qiagen, 51104)

DNA samples with concentration in range from 20 to 200 ng/μl could be analyzed.

WARNINGS AND PRECAUTIONS

1. RUO – Research Use Only.
2. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
3. Do not pipette by mouth.
4. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
5. Do not use a kit after its expiration date.
6. Do not mix reagents from different kits.
7. Dispose all specimens and unused reagents in accordance with local regulations.
8. Specimens and controls should be prepared in a laminar flow hood.
9. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
10. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
11. Specimens may be infectious. Use Universal Precautions when performing the assay.
12. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant. Follow by wiping down the surface with 70% ethanol.
13. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
14. Material Safety Data Sheets (MSDS) are available on request.
15. Use only recommended nucleic acid isolation kits for sample preparation.
16. Use of this product should be limited to personnel trained in the techniques of PCR.
17. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification Area. Do not return samples, equipment and reagents in the area where you performed previous step. Personnel should be using proper anti-contamination safeguards when moving between areas.

STORAGE INSTRUCTIONS

Store kit at -20°C. The kit can be shipped at 2-8°C for 3-4 days but should be stored at -20°C immediately on receipt.

STABILITY

FV Leiden G1691A SNP-Screen is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

The complete kit has been tested on a SmartCycler® (Cepheid).

Certificates of Analyses are available on request at info@sacace.com

SAMPLE COLLECTION, STORAGE AND TRANSPORT

FV Leiden G1691A SNP-Screen can analyze genomic DNA extracted from:

- *whole blood* collected in EDTA tubes;

Specimens can be stored at +2-8°C for no longer than 48 hours, or freeze at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

PROTOCOL

FV Leiden G1691A SNP-Screen kit doesn't include reagents required for sample preparation and DNA extraction. Blood samples and biological material must undergo stages of samples preparation and DNA extraction by the recommended kits or those similar by quality and quantity of the extracted DNA. Use of blood samples collected in tubes containing heparin is not recommended.

The analysis of the genomic DNA specimens using **FV Leiden G1691A SNP-Screen** kit includes the following stages:

1. Preparing the Real Time PCR;
2. Real Time PCR analysis;
3. Data analysis with the software of Real Time PCR instrument;
4. Results analysis and conclusions.

EXPERIMENTAL PROTOCOL

1. Prepare required quantity of SmartCycler reaction tubes for samples and controls.
2. Thaw reagents, vortex and centrifuge briefly.
3. Calculate the required volume of the components, based on the quantity of the samples plus 4 (3 positive controls and 1 negative control). Prepare in the new sterile tube **10*N µl of PCR Master Mix, 10*N of Diluent and 0,5*N of Taq DNA Polymerase**. Vortex and centrifuge briefly.

N	Reagent	Reaction Volume, µl	×5	×10	×16
1	PCR Master Mix	10	50	100	160
2	Diluent	10	50	100	160
3	Taq DNA Polymerase	0,5	2,5	5	8

4. Add to each tube **20 µl of Reaction Mix**.
5. Add **5 µl of extracted DNA** to appropriate SmartCycler tube. Mix by pipetting in the highest part of the tube.
6. Prepare for each panel 4 controls:
 - add **5 µl** of **C-** to the tube labeled Amplification Negative Control;
 - add **5 µl** of **Pos1, Pos2 and Pos3** to the tubes labeled as Positive Controls;
7. Transfer the tubes into the rotor of the minicentrifuge and centrifuge briefly.
8. Insert the tubes in the thermalcycler.
9. The Real Time PCR reaction and the interpretation of the results must be performed in accordance to the instructions in the Operator's Manuals.

Real Time Amplification with SmartCycler® (Cepheid)

DEFINE PROTOCOLS

1. Open the program of the instrument and select in the main menu **Define Protocols**



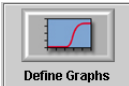
2. Press the button **New Protocol**.
3. In the window enter the name of protocol – **SNP-Screen** and press **OK**.
4. Set the amplification program with the following parameters:

Stage 1			Stage 2			
Hold			Repeat 40 times.			
Temp	Secs	Optics	5-Temperature Cycle			
92.0	120	Off	Deg/Sec	Temp	Secs	Opti...
			NA	92.0	10	Off
			NA	62.0	10	Off
			NA	63.5	5	Off
			NA	65.0	5	Off
			NA	50.0	10	On
			<input type="checkbox"/> Advance to Next Stage			

5. Click **Save Protocol**

DEFINE GRAPHS

1. Press the button **Define Graphs**



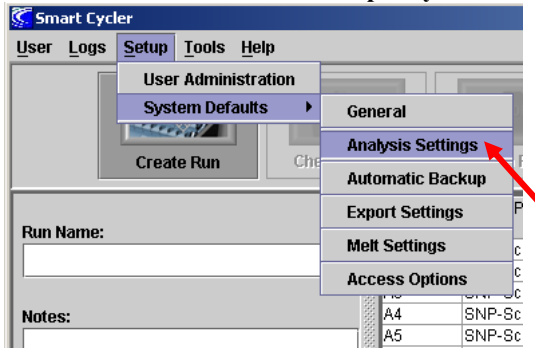
2. In the opened window click **New Graph**.
3. In the new window enter the name of graphic – **SNP-Screen**.
4. Choose **Automatically added to new Runs**, in the window **Graph Type** select **Optics** and the following parameters:

<input checked="" type="checkbox"/> Automatically added to new Runs
Graph Type: Optics
Channel(s):
<input checked="" type="checkbox"/> Ch 1
<input checked="" type="checkbox"/> Ch 2
<input type="checkbox"/> Ch 3
<input type="checkbox"/> Ch 4
Show:
<input checked="" type="checkbox"/> Primary Curve
<input type="checkbox"/> 2nd Derivative
<input checked="" type="checkbox"/> Threshold (Horizontal)
<input type="checkbox"/> Threshold Crossings (Vertical)
Axes:
<input checked="" type="radio"/> Fluorescence vs. Cycle
<input type="radio"/> Log Fluorescence vs. Cycle

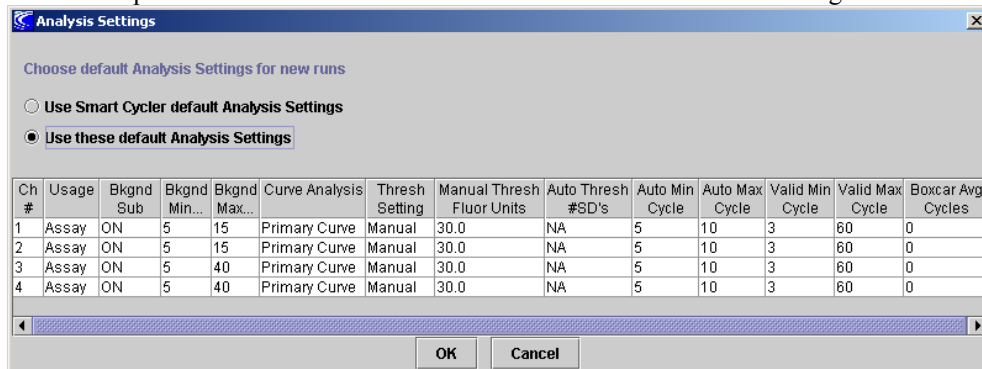
5. Click **Save Graph**.

ANALYSIS SETTINGS

1. Select in the main menu **Setup**→**System Defaults**→**Analysis Settings**.



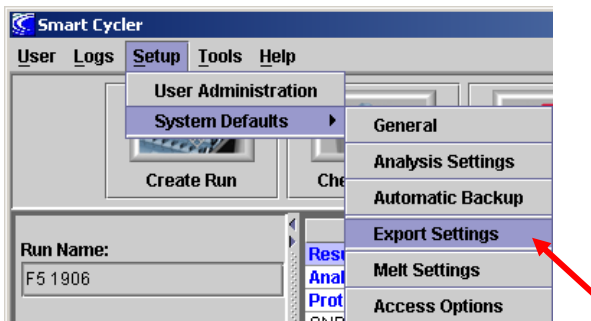
2. In the opened window select **Use these defaults...** and enter the following dates:



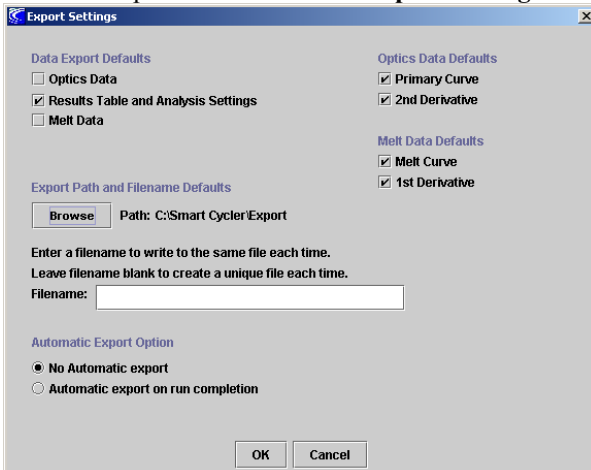
3. Press **OK**.

EXPORT SETTINGS

1. Select in the main menu **Setup**→**System Defaults**→**Export Settings**



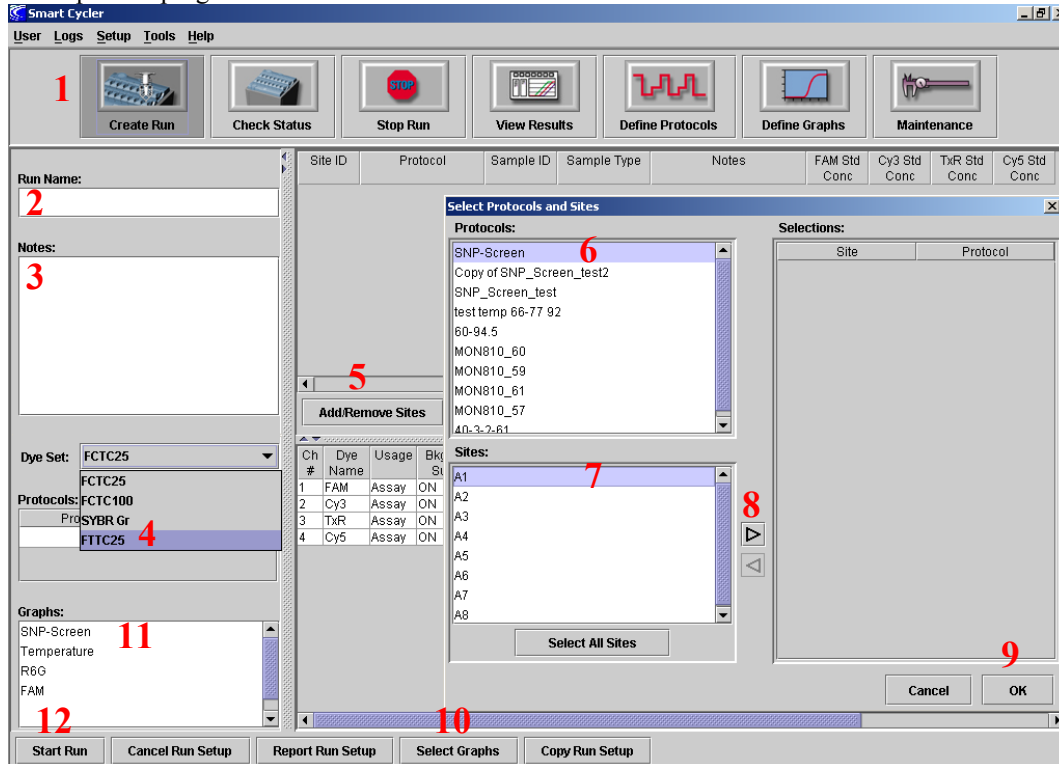
2. In the opened window select **Export Settings** and enter the following settings:



3. Press **OK**

STARTING THE PCR RUN

1. Open the program of the instrument.

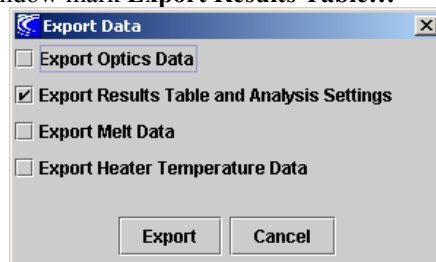


2. Press **Create Run (1)**. Fill the **Run Name (2)** and **Notes (3)** fields if necessary.
3. Select **FTTC25** in the field **Dye Set (4)**.
4. Click **Add/Remove Sites (5)** and select in the new window **Protocols**, the protocol **SNP-Screen (6)**.
5. Select in the field **Sites (7)** the necessary quantity of cells and press the right position indicator **(8)**.
6. Click **OK (9)**.
7. Press the button **Select Graphs (10)** and select in the opened window the following graphs:

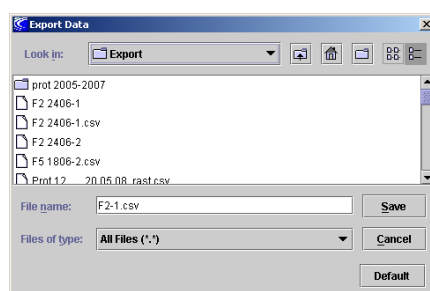
Selected Graphs:	
SNP-Screen	
Temperature	
R6G	
FAM	
8. Press **OK**. The selected graphs appears in the window **Graphs (11)**. Press **Start Run (12)**.

EXPORT RESULTS

1. After the end of the experiment press the button **Export**.
2. In the opened window mark **Export Results Table...**



3. Click **Export**.



4. Click **Save**.

Results Interpretation

Results of Controls:

1. The result of the *Pos1 Control*, DNA “FV(-/-)” (normal genotype FV G/G analysis must correspond to the following conditions:
 - The reaction result must be positive in the channel Fam and negative in the channel R6G.
 - The threshold cycle value for the Fam channel must be less than 31.
2. The result of the *Pos2 Control*, DNA “FV (+/-)” (heterozygous mutation FV G/A) analysis must correspond to the following conditions:
 - The reaction result must be positive in the Fam and R6G channels.
 - The threshold cycle value for the Fam and R6G channels must be less than 33.
3. The result of the *Pos3 Control*, DNA “FV(+/+)” (homozygous mutation FV A/A) analysis must correspond to the following conditions:
 - The reaction result must be positive in the R6G channel and negative in the FAM channel.
 - The threshold cycle value for the R6G channel must be less than 31.
4. The result of *Neg Control* analysis must be negative in the Fam and R6G channels.
In case of positive results for Neg Control on one of the channels, the results of the experiment are considered invalid. It is required to take measures to detect and eliminate the source of contamination and to repeat the analysis.
5. The analysis' results of the samples are acceptable only if all the above mentioned conditions for the control samples are met.

Results of samples

The condition of human genomic DNA in position 1691 of FV gene in the sample is considered:

1. Homozygous normal (FV G/G) if there is a positive result on the Fam channel, a negative result on the R6G channel and the threshold cycle value on the Fam channel is less than ($Ct_{POS1} + 1$);
2. Heterozygous by mutation (FV G/A) if there is a positive result on the Fam channel, a positive result on the R6G channel and the threshold cycle value on these channels is less than ($Ct_{POS2} + 1$);
3. Homozygous by mutation (FV A/A) if there is a positive result on the R6G channel, a negative result on the Fam channel and the threshold cycle value on the R6G channel is less than ($Ct_{POS3} + 1$);
4. Not valid if there are negative results on the Fam and R6G channels.

Key to symbols used



Catalogue Number



Research Use Only



Lot Number



Expiration Date



Contains reagents



Caution!



Version



Manufacturer



Temperature limitation



Sacace Biotechnologies Srl
18 San Carlo str., 81100 Caserta, Italy

*PCR: The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-La Roche and applicable in certain countries. Sacace does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this kit is recommended for persons that either have a license to perform PCR or are not required to obtain a license