

FACTOR V LEIDEN KIT

For use with the LightCycler® 2.0 Instrument

REF 03 610 179 001

Kit for 32 reactions for a maximum of 30 specimens

For *in vitro* diagnostic use.

IMPORTANT NOTE:

LOT SPECIFIC INFORMATION FOR THE FACTOR V LEIDEN KIT HAS BEEN SUPPLIED ON THE BACK OF THIS PACKAGE INSERT AS A BARCODE OR IN ALPHANUMERIC FORM. THIS LOT SPECIFIC INFORMATION MUST BE ENTERED INTO THE LIGHTCYCLER® 2.0 INSTRUMENT SOFTWARE EITHER MANUALLY OR BY SCANNING THE BARCODE DURING THE WORKFLOW. IT IS IMPORTANT TO ENSURE THAT THE LOT NUMBER ON THE BACK OF THIS INSERT AND THE LOT NUMBER ON THE KIT CARTON ARE IDENTICAL BEFORE ENTERING THE INFORMATION.

INTENDED USE

The Factor V Leiden Kit allows the detection and genotyping of a single point mutation (G to A at position 1691) of the human Factor V gene, referred to as Factor V Leiden mutation, from DNA isolated from human whole peripheral blood. The test is performed on the LightCycler® 2.0 Instrument utilizing polymerase chain reaction (PCR) for the amplification of Factor V DNA recovered from clinical specimens and fluorogenic target-specific hybridization for the detection and genotyping of the amplified Factor V DNA.

The Factor V Leiden test is an *in vitro* diagnostic test for the detection and genotyping of the Factor V Leiden mutation as an aid to diagnosis in the evaluation of patients with suspected thrombophilia. The test is intended to be used on the LightCycler® 2.0 Instrument using the LightCycler® Software 4.05 or 4.1. The specimen preparation must be performed according to the workflow procedures described below.

EXPLANATION OF THE TEST

Inherited thrombophilia predispose an individual to thrombotic events such as venous thrombosis, the third most common cardiovascular disease (1). Activated protein C (APC) resistance is regarded as the most prevalent coagulation abnormality associated with venous thrombosis (1,2). A point mutation at position 1691 of the Factor V gene, referred to as Factor V Leiden mutation, causes an arginine to glutamine substitution at position 506 in the Factor V protein and renders it partially resistant to inactivation by APC. Genetic analysis has demonstrated that this mutation, which has a relatively high prevalence in the general population (*e.g.*, about 5% in Caucasians), accounts for 85% to 95% of APC resistance cases (2).

TEST PRINCIPLE / SUMMARY

Specimen Preparation

The specimen preparation can be performed either manually or automated by using the High Pure PCR Template Preparation Kit or the MagNA Pure LC Instrument, running the MagNA Pure LC DNA Isolation Kit I. These are the only purification methods validated for use with the Factor V Leiden test.

Detection

1. A 222 bp fragment of the Factor V gene is amplified from human genomic DNA using specific primers.
2. The amplicon is detected by fluorescence using a specific pair of HybProbe probes. The HybProbe probes consist of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the PCR cycle. One probe is labeled at the 5'-end with LightCycler® Red 640-N-hydroxy-succinimide ester (Red 640-NHS ester), and to avoid extension, modified at the 3'-end by phosphorylation. The other probe is labeled at the 3'-end with fluorescein.
3. Only after hybridization to the template DNA, do the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, fluorescein, the donor fluorophore, is excited by the light source of the LightCycler® 2.0 Instrument, and part of the excitation energy is transferred to LightCycler® Red 640-NHS ester, the acceptor fluorophore.
4. The emitted fluorescence of LightCycler® Red 640-NHS ester is then measured by the LightCycler® 2.0 Instrument.

Genotyping

The HybProbe probes are also used to determine the genotype by performing a melting curve analysis after the amplification cycles are completed and the amplicon is present at increased concentration.

- The Red 640-labeled HybProbe probe hybridizes to a part of the target sequence that is not mutated and functions as an anchor probe.
 - The Fluorescein-labeled HybProbe probe spans the mutation site (mutation probe).
- During the melting curve analysis, increasing temperature causes the fluorescence to decrease because the shorter of the two probes (mutation probe) dissociates first and the two fluorescent dyes are no longer in close proximity. If the Factor V Leiden mutation is present, the mismatch of the mutation probe with the target destabilizes the hybrid so the decrease in fluorescence will occur at a lower temperature. With the wild-type genotype, mismatches will not occur, and therefore, the heteroduplex DNA has a higher melting temperature (T_m). The heterozygous genotype exhibits a distinctive combination of properties.



REAGENTS – WORKING SOLUTIONS

1. FVL MD Mix (Factor V Leiden Mutation Detection Mix) 1 × 78 µL	<ul style="list-style-type: none"> • <0.01% FVL primer forward and reverse • <0.01% FVL HybProbe probe Red 640 • <0.01% FVL HybProbe probe Fluorescein 	<ul style="list-style-type: none"> • Brij 35 • MgCl₂
2. FVL R Mix (Factor V Leiden Reaction Mix) 1 × 78 µL	<ul style="list-style-type: none"> • Tris-HCl buffer • <0.01% Taq DNA polymerase, GMP grade • <0.07% dATP, dCTP, dGTP, dUTP, dTTP 	<ul style="list-style-type: none"> • Brij 35 • MgCl₂
3. FVL CT (Factor V Leiden Control Template) 1 × 50 µL	<0.01% control template DNA contains plasmid: pCRF5 WT and pCRF5MUT	
4. FVL DIL (Factor V Leiden Diluent) 1 × 1 mL	H ₂ O, PCR-grade (also used as negative control for amplification)	

PRECAUTIONS AND WARNINGS

Polymorphism

Within the Factor V gene, mutations in addition to the FVL mutation at position 1691 exist, and these additional mutations are spanned by the mutation probe. Three rare mutations will lead to a false-positive result (*i.e.*, het or mut genotype) after performing the melting curve analysis (*i.e.*, mutations A1692C, G1689A, and A1696G).

Note: The Factor V Leiden test can be performed in addition or subsequently to a functional assay detecting the phenotypic activated protein C (APC) resistance. Only the FVL mutation is linked to APC resistance, not the three rare mutations listed above.

Handling Requirements

- **FOR IN VITRO DIAGNOSTIC USE. Use of this product should be limited to personnel trained in the techniques of PCR.**
- Exercise the normal precautions required for handling all laboratory reagents.
- This test is for use with human whole blood collected in EDTA or citrate anticoagulants only. **Heparin might interfere with the PCR and must not be used with this procedure.**
- Laboratory workflow must be in accordance with Good Laboratory Practice (GLP), and in addition, proceed in a uni-directional manner (*i.e.*, specimen preparation, set-up of the PCR, and the PCR run with the LightCycler® 2.0 Instrument must be physically separated).
- Do not pool reagents from different lots or from different bottles of the same lot. Immediately after use, close all bottles in order to avoid leakage, varying buffer-concentrations or buffer-conditions. After opening, store all bottles and vials in an upright position.
- Do not mix reagents from different lots.
- Do not use a kit after its expiration date.
- Avoid contact of the Lysis/Binding Buffer and the Wash Buffer I from the MagNA Pure LC DNA Isolation Kit I or the Binding Buffer and the Inhibitor Removal Buffer from the High Pure PCR Template Preparation Kit with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If the reagent spills, dilute with water before wiping dry.
- Do not allow the Lysis/Binding Buffer from the MagNA Pure LC DNA Isolation Kit I, which contains guanidine thiocyanate, to come in contact with sodium hypochlorite (bleach) solution or strong acidic solutions. This mixture can produce a highly toxic gas.

Laboratory Procedures

- All human sourced material and all resulting waste should be considered potentially infectious. Thoroughly clean and disinfect all work surfaces with disinfectants recommended by the local authorities.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection when handling specimens and kit reagents.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes is recommended.
- Wash hands thoroughly after handling specimens and test reagents.

Waste Handling

- Dispose of unused reagents and waste in accordance with country, federal, state, and local regulations.
- Material Safety Data Sheets (MSDS) are available upon request from the local Roche office.

Specimen Preparation

- Refer to the safety instructions in the package insert of the MagNA Pure LC DNA Isolation Kit I or the High Pure PCR Template Preparation Kit for handling and disposal information.
- The MagNA Pure LC DNA Isolation Kit I or the High Pure PCR Template Preparation Kit must be stored at room temperature (+15°C to +25°C). Before using the Lysis/Binding Buffer in the MagNA Pure LC DNA Isolation Kit I, shake to dissolve precipitates. Do not store below room temperature.
- Use only MagNA Pure LC Medium Reagent Tub 20 (Cat. No. 03 004 058 001) or MagNA Pure LC Reagent Tub (large) (Cat. No. 03 004 040 001) at the respective positions indicated by the MagNA Pure LC Instrument with this procedure.

Amplification and Detection

- Consult the LightCycler® 2.0 Instrument Operator's Manual for IVD use before using this kit.
- Create a written record correlating with the LightCycler® Carousel ID. The record must contain the LightCycler® Sample Carousel position, capillary number, and name associated with each specimen to ensure correct specimen identification.
- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.

REAGENT HANDLING

1. FVL MD Mix (yellow cap, vial 1), ready-to-use	<ul style="list-style-type: none"> • Store at -15°C to -25°C. • Keep away from light!
2. FVL R Mix (red cap, vial 2), ready-to-use	<ul style="list-style-type: none"> • Store at -15°C to -25°C.
3. FVL CT (purple cap, vial 3), ready-to-use	<ul style="list-style-type: none"> • Store at -15°C to -25°C
4. FVL DIL (colorless cap, vial 4), ready-to-use	<ul style="list-style-type: none"> • Store at -15°C to -25°C

STORAGE / STABILITY (REAGENTS)

- The unopened kit should be stored at -15°C to -25°C until the expiration date printed on the label.
- Keep the FVL MD Mix (vial 1, yellow cap) away from light!
- Thaw the components of the Factor V Leiden Kit, mix gently, and store on ice.
- Freeze immediately after use.
- Kit reagents may be frozen and thawed up to five times.

SPECIMEN COLLECTION / PREPARATION AND STABILITY

For preparation of genomic DNA from human blood, perform the nucleic acid purification using the High Pure PCR Template Preparation Kit (Cat. No. 11 796 828 001) or the MagNA Pure LC DNA Isolation Kit I (Cat. No. 03 003 990 001) in combination with the MagNA Pure LC Instrument (Cat. No. 12 236 931 001) as described below.

- EDTA or citrate anti-coagulated human peripheral blood is stable for at least 7 days at +2°C to +8°C. It may be stored frozen at -20°C for up to 12 months and thawed once.
- Eluted DNA can be stored in a sealed Sample Cartridge or in Sarstedt screw cap tubes at +2°C to +8°C for up to 7 days. It is stable for long term storage (approximately 3 weeks) at -15°C to -25°C. Eluted DNA may be frozen and thawed up to three times.

MATERIALS PROVIDED

See “Reagents – working solutions” on page 2.

MATERIALS AND DEVICES REQUIRED BUT NOT PROVIDED

- LightCycler® 2.0 Instrument (Cat. No. 03 531 414 201)
Note: For the USA, different catalog numbers do apply. Please contact the US sales office for details.
(Serial number 1415001 and higher are approved for IVD use and use with this kit. Instruments with a serial number below 1415001 are also approved for use with this kit after they have been approved for IVD usage via a special upgrade procedure. Please contact your local customer support for details on the upgrade procedure.)
- LightCycler® Software 4.05 (Cat. No. 04 717 392 001) or 4.1 (Cat. No. 04 898 915 001)
- LightCycler® Capillaries (20 µL) (Cat. No. 04 929 292 001)
- High Pure PCR Template Preparation Kit (Cat. No. 11 796 828 001)
- Microcentrifuge Tubes, 1.5 mL, sterile, nuclease free
- LightCycler® Centrifuge Adapters (Cat. No. 11 909 312 001)
- Barcode Reader (Cat. No. 04 557 174 001)
- Factor V Leiden Kit Macro (Cat. No. 04 586 913 001)
- Ethanol p.a.
- Isopropanol p.a.
- H₂O, double distilled, sterile, nuclease-free
- MagNA Pure LC Instrument (Cat. No. 12 236 931 001)
- MagNA Pure Software 3.011 or higher (Cat. No. 03 322 025 001)
- MagNA Pure LC DNA Isolation Kit I (Cat. No. 03 003 990 001)
- LC Carousel Centrifuge 2.0 plus rotor buckets [Cat. No. 03 709 582 001 (230 V) or Cat. No. 03 709 507 001 (115 V)]. If you have already purchased a LC Carousel Centrifuge [Cat. No. 12 189 682 001 (230 V) or Cat. No. 03030 512 001 (115 V)], a LightCycler® Carousel 2.0 rotor set (Cat. No. 03 724 697 001)* is also required.
- Sarstedt screw-cap tubes, 1.5 mL, sterile
- * See LightCycler® 2.0 Instrument Operator’s Manual, Software Version 4.05 or 4.1 for *in vitro* diagnostic use, or contact Technical Support for equivalent centrifuge specifications if a LC Carousel Centrifuge is unavailable. In this case, LightCycler® Centrifuge Adapters are needed as well (Cat. No. 11 909 312 001)

OPTIONAL MATERIALS

- Thrombophilia Post-Elution Protocols CD (Cat. No. 04 378 784 001)
- MagNA Pure LC Barcode Reader Quick Scan 1000 (Cat. No. 03 186 580 001)
- MagNA Pure LC Barcode Printer TLP 2844 (Cat. No. 03 576 094 001)
- MagNA Pure LC Barcode Labels (Cat. No. 03 531 520 001)
- MagNA Pure LC Barcode Printer Ribbon (Cat. No. 03 531 538 001)
- MagNA Pure LC Cooling Block, LC Sample Carousel (Cat. No. 12 189 704 001)
- LightCycler® Capping Tool (Cat. No. 03 357 317 001)
- General laboratory equipment

ASSAY PROCEDURE

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- ① **Automated specimen preparation procedure for 15 or 30 specimens using the MagNA Pure LC DNA Isolation Kit I (Cat. No. 03 003 990 001) in combination with the MagNA Pure LC Instrument (Cat. No. 12 236 931 001)**

A. PURIFICATION

Before you begin

Start precooling the MagNA Pure LC Cooling Block, LC Sample Carousel (Cat. No. 12 189 704 001) in a refrigerator at +2°C to +8°C (for at least 2 hours).

Starting the MagNA Pure LC Instrument and Software

1. Turn on the MagNA Pure LC Instrument, then start the computer.
2. Start the MagNA Pure LC Software, then at the **Main Menu** screen, click on the **Sample Ordering** button.
3. At the **Sample Ordering** screen, select the protocol titled, *DNA / Blood Cells Fast* Protocol, then enter the lot numbers of the MagNA Pure LC DNA Isolation Kit I (Cat. No. 03 003 990 001) and the Factor V Leiden Kit and enter the LightCycler® Carousel ID (do not change other settings).
4. Enter the negative control (FVL DIL, vial 4, colorless cap) in row 1 of the Sample Order table (position A1) by typing “Negative Control”.
5. Starting in row 1 (position B1), type in the specimen names or use the MagNA Pure LC Barcode Reader (Optional). Enter 50 µL as the

- Sample Volume and 0 μL as the Dilution Volume (preset). The Elution volume is preset to 100 μL .
- Save and print the Sample Order file information.
 - Use the **Print Sample Names** function to produce a printout showing the specimen names and their correct position in the Sample Cartridge. (Optional)
 - Print **Cartridge Barcode** labels in triplicate. (Optional)
 - Label Sample Cartridge (for elution) and Printouts with the barcode labels. (Optional)
 - Click on **Stage Setup**.

Preparation of Proteinase K working solution

(sufficient for 32 specimens)

- Add 3 mL Elution Buffer (bottle 6, yellow cap) to one vial of Proteinase K, lyophilized (vial 4, pink cap). After the Proteinase K is completely dissolved, add an additional 2 mL of the Elution Buffer to reach a final volume of 5 mL.
- Close the vial and mix well.

Note: If the reconstituted solution is not used the same day, store at +2°C to +8°C in vial 4 (up to 4 weeks).

All other reagents are ready-to-use.

Loading of reagents and disposable plastics

Note: Do not use the MagNA Pure LC Tub 30. Use only MagNA Pure LC Medium Reagent Tub 20 (Cat. No. 03 004 058 001) or MagNA Pure LC Reagent Tub (large) (Cat. No. 03 004 040 001) at the respective positions indicated by the MagNA Pure LC Instrument with this procedure.

- The status of the **Heat Unit** and the **Cool Units 1 & 2** must be displayed as **PASS** before filling the respective Reagent Tub with the Magnetic Glass Particles (MGP) suspension.
- Manually fill the Reagent Tubs (outside the MagNA Pure LC Instrument) with the volumes indicated on the **Start Information** screen (by the **Stage Layout** graphic), then cap them with the corresponding Reagent Tub Lids and Tub Lid Seals. Place the filled Reagent Tubs into the Reagent Reservoir Rack at the positions indicated on the **Start Information** screen (except the MGP suspension, see step 4).
 - Place the additional necessary disposable plastics (as indicated) and the prepared Reagent Reservoir Rack on the Reagent/Sample Stage according to the information on the **Start Information** screen.
 - Manually transfer 50 μL resuspended EDTA or citrate anti-coagulated human whole peripheral blood specimens directly into the Sample Cartridge (outside the MagNA Pure LC Instrument) according to sample order list.
 - Immediately before starting the run, vortex the MGP glass vial thoroughly, fill a Reagent Tub with the MGP suspension, cap it with the corresponding Reagent Tub Lid, and place it onto the Instrument Reagent/Sample Stage in the position specified on the **Start Information** screen.
 - Place the Sample Cartridge into the MagNA Pure LC Instrument.

Purification Run

- Confirm every loaded position on the MagNA Pure LC Instrument via mouse click on the screen (**Start Information** screen). After all items are confirmed, the **OK** button will appear.
- Remove the Tub Lid Seals and close the lock bar and the door of the MagNA Pure LC Instrument.
- Click the **OK** button to start the run. The **Batch Status** screen appears and a purple line moving from left to right indicates the actual progress of the isolation procedure.
- The **Result** screen will appear after the run is completed.
- Save and print the **Result** screen (optional).
- Optionally, select the Open Post-Elution function on the Action menu. Otherwise, proceed to step B of Chapter 2 (manual specimen preparation) of this package insert.
- If the MagNA Pure extraction fails for an individual specimen (noted on the **Result** screen) the respective specimen must be re-extracted.

If the Post-Elution function cannot be performed, proceed to step B of Chapter 2 (manual specimen preparation) of this package insert using the specimens that were initially extracted or re-extracted successfully.

B. POST-ELUTION (Optional)

Post-Elution Procedure (Optional)

- Ensure the MagNA Pure LC Cooling Block, LC Sample Carousel is in place and has been precooled (e.g., refrigerate at +2°C to +8°C for at least 2 hours).
- Load the Post-Elution Protocol titled *Thrombophilia 15 or 30 samples* from the Thrombophilia Post-Elution Protocols CD (Cat. No. 04 378 784 001). (Optional)
- Master Mix preparation (for Post-Elution procedure only)
 - Only prepare the required amount immediately prior to use.
 - Thaw the components of the Factor V Leiden Kit, mix gently, and store on ice.
 - Place one LightCycler[®] Capillary per DNA specimen and one for each of the controls in the LightCycler[®] Carousel, i.e., 17 capillaries for 15 specimens or 32 capillaries for 30 specimens, respectively.
 - Prepare the Master Mix:

Step	Action															
1	In a 1.5 mL Sarstedt screw-cap tube on ice, add the following components in the order listed below (Examples given for 15 or 30 specimens): <table border="1" data-bbox="232 1641 1439 1786"> <thead> <tr> <th>Reagents-working solutions</th> <th>Volume* / 15 specimens</th> <th>Volume* / 30 specimens</th> </tr> </thead> <tbody> <tr> <td>FVL DIL, vial 4</td> <td>209 μL</td> <td>396 μL</td> </tr> <tr> <td>FVL MD Mix, vial 1</td> <td>38 μL</td> <td>72 μL</td> </tr> <tr> <td>FVL R Mix, vial 2</td> <td>38 μL</td> <td>72 μL</td> </tr> <tr> <td>Total volume</td> <td>285 μL</td> <td>540 μL</td> </tr> </tbody> </table> <p>* volumes indicated include controls and pipetting margins</p>	Reagents-working solutions	Volume* / 15 specimens	Volume* / 30 specimens	FVL DIL, vial 4	209 μL	396 μL	FVL MD Mix, vial 1	38 μL	72 μL	FVL R Mix, vial 2	38 μL	72 μL	Total volume	285 μL	540 μL
Reagents-working solutions	Volume* / 15 specimens	Volume* / 30 specimens														
FVL DIL, vial 4	209 μL	396 μL														
FVL MD Mix, vial 1	38 μL	72 μL														
FVL R Mix, vial 2	38 μL	72 μL														
Total volume	285 μL	540 μL														
2	<ul style="list-style-type: none"> Mix gently. Ensure that no air bubble is trapped in the tip of the tube. 															
3	Place the tube containing the Master Mix on the MagNA Pure LC cooling block at position 1. Remove the tub lid.															

- Place the LightCycler[®] Carousel, including the capillaries indicated by the Post-Elution Protocol, into the cooling block and place the

required number of pipette tips as indicated on the screen on the stage of the MagNA Pure LC Instrument.

- Open the FVL CT (vial 3, purple cap), pipette 25 µL into a new 1.5 mL Sarstedt screw-cap tube and then place it on the MagNA Pure LC cooling block at position 16 as indicated by the Post-Elution Protocol.

Note:

- Centrifuge the vial containing the FVL CT to spin down its contents prior to opening.
- Ensure that no air bubble is trapped in the tip of the tube.
- Change gloves after handling the control template.

- Close the instrument, then click on **Start** and **OK** to begin automated pipetting. Once the Post-Elution has finished, the **Post-Elution Result** screen appears.
- Print the Cool Block barcode in triplicate. (Optional)
- Save** and **Print Post-Elution** result table, then label the printout with a Cool Block barcode. (Optional)
- Label an empty diskette with the name of the purification run or a Cool Block barcode. Generate a LightCycler® SAM file (Sample Pattern File) on the diskette.
- Seal each capillary with a stopper using the LC Capping Tool (Cat. No. 03 357 317 001).
- Label the LightCycler® Carousel with a Cool Block barcode. (Optional)
- Place the LightCycler® Carousel into the LC Carousel Centrifuge rotor bucket.
- Transfer the rotor bucket, including the LightCycler® Carousel, to the LC Carousel Centrifuge.

Note: If no LC Carousel Centrifuge is available, use a benchtop centrifuge (like the Biofuge® 13 from Heraeus Instruments or Kendro Laboratory Products) with LightCycler® Centrifuge Adapters and spin at max. 735 × g for 30 s.
- Press **Start** to commence the LC Carousel Centrifuge run.
- After centrifugation, transfer the LightCycler® Carousel to the LightCycler® 2.0 Instrument.

Note: Store the eluted DNA at +2°C to +8°C for up to seven days and at -15°C to -25°C for long term storage (approximately 3 weeks). If not using the Post-Elution Protocol with the MagNAPure LC, proceed to step B in the Chapter 2 protocol for PCR Master Mix set-up.
- If no further MagNA Pure runs are to be performed, shut down the MagNA Pure LC computer and the MagNA Pure LC Instrument.

C. AMPLIFICATION AND DETECTION

LightCycler® 2.0 Instrument

Note: Before starting the Macro, log onto an Exor Database (DB) with an audit trail. If logged onto the Exor DB with an audit trail, the word "Traceable" is displayed in the Status bar of the software. For further information on how to login to the software, refer to the LightCycler® 2.0 Instrument Operator's Manual, Software Version 4.05 or 4.1 for *in vitro* diagnostic use.

- If previously labeled (Step B.11), remove the barcode label from the LightCycler® Carousel. (Optional)
- Place the carousel into the LightCycler® 2.0 Instrument.
- Make sure that the Factor V Leiden Kit Macro (Cat. No. 04 586 913 001) is installed. This Macro only needs to be installed once when performing the test for the first time.
- Start the macro by pushing the Roche button and entering the Cat. No. of the kit, either manually or by scanning the barcode. Refer to the back of this package insert for the correct kit specific barcode information.

Note: "Perform self test" may not be active.
- The kit wizard will guide you through the entire procedure.
- Enter specimen names in the **Capillary View** screen either manually or, if previously created, using a SAM-file by pressing "Import SAM". Enter the Kit lot number in the respective field on the **Capillary View** screen.
- Click "Start Run" button within the kit wizard.
- Once the run is complete, the LightCycler® 2.0 Instrument automatically determines and displays the genotype by performing a melting curve analysis.

Note: The fluorescence signal is measured in the 640 nm channel.

② **Manual specimen preparation procedure for one specimen using the High Pure PCR Template Preparation Kit (Cat. No. 11 796 828 001)**

A. PURIFICATION

Before you begin

Start precooling the LightCycler® Centrifuge Adapters in a refrigerator at +2°C to +8°C (for at least 2 hours).

Preparation of working solutions

Reagent	Reconstitution/Preparation of working solution	Storage and stability of working solution
Proteinase K (vial 3, pink cap)	Dissolve Proteinase K in 4.5 mL double distilled water, aliquot solution.	Store at -15°C to -25°C. Stable for 12 months.
Inhibitor Removal Buffer (vial 4a, black cap)	Add 20 mL ethanol to Inhibitor Removal Buffer. Note: Mark and date bottle once ethanol has been added.	Store at +15°C to +25°C. Stable until expiration date printed on kit label.
Wash Buffer (vial 4, blue cap)	Add 80 mL ethanol to Wash Buffer. Note: Mark and date bottle once ethanol has been added.	Store at +15°C to +25°C. Stable until expiration date printed on kit label.

Purification procedure

Note: Make sure to exactly follow the procedure described below and not the procedure described in the package insert of the High Pure PCR Template Preparation Kit.

- Aliquot 200 µL Elution Buffer per specimen into sterile 1.5 mL reaction tubes, close the tubes, and prewarm to +70°C.
- To 200 µL resuspended EDTA or citrate anti-coagulated human whole peripheral blood add:
 - 200 µL Binding Buffer (green cap)
 - 40 µL Proteinase K (reconstituted)
 - Mix immediately by vortexing and incubate at +70°C for 10 min.
- Add 100 µL isopropanol and mix well by vortexing.
- Pipette the specimen mixture into the upper reservoir of a combined High Pure filter tube-collection tube assembly.
- Centrifuge for 1 min at 6000 × g in a standard tabletop centrifuge.
- Discard the flowthrough and collection tube.
- Combine the filter tube with a new collection tube.
- Add 500 µL Inhibitor Removal Buffer (black cap) to the upper reservoir.

9. Centrifuge for 1 min at 6000 × g.
 10. Discard the flowthrough and collection tube.
 11. Combine the filter tube with a new collection tube.
 12. Add 500 µL Wash Buffer (blue cap) to the upper reservoir.
 13. Centrifuge for 1 min at 6000 × g.
 14. Discard the flowthrough and collection tube.
 15. Combine the filter tube with a new collection tube.
 16. Add 500 µL Wash Buffer (blue cap) to the upper reservoir.
 17. Centrifuge for 1 min at 6000 × g.
 18. Discard the flowthrough.
 19. Combine the filter tube and the same collection tube.
 20. Centrifuge for 10 s at max. speed (13,000 × g or higher) to remove residual Wash Buffer.
 21. Discard the collection tube.
 22. Insert the filter tube into a clean 1.5 mL reaction tube.
 23. Add the 200 µL prewarmed (+70°C) Elution Buffer to the filter tube.
 24. Centrifuge for 1 min at 6000 × g.
- The microfuge tube contains the eluted DNA.

B. MASTER MIX PREPARATION

1. Thaw the components of the Factor V Leiden Kit, mix gently, and store on ice.
 2. Place one LightCycler® Capillary per DNA specimen and one for each of the controls into precooled LightCycler® Centrifuge Adapters.
 3. Prepare the Master Mix by multiplying the amount in the "Volume/reaction" column by the number of reactions to be run (*i.e.*, specimen DNAs, negative and positive controls), plus one additional reaction.
- Proceed as described below for a 20 µL standard reaction.

Step	Action										
1	In a 1.5 mL reaction tube on ice, add the following components in the order listed below: <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 70%;">Reagents–working solutions</th> <th style="width: 30%;">Volume/reaction</th> </tr> </thead> <tbody> <tr> <td>FVL DIL, vial 4</td> <td>11 µL</td> </tr> <tr> <td>FVL MD Mix, vial 1</td> <td>2 µL</td> </tr> <tr> <td>FVL R Mix, vial 2</td> <td>2 µL</td> </tr> <tr> <td>Total volume</td> <td>15 µL</td> </tr> </tbody> </table>	Reagents–working solutions	Volume/reaction	FVL DIL, vial 4	11 µL	FVL MD Mix, vial 1	2 µL	FVL R Mix, vial 2	2 µL	Total volume	15 µL
Reagents–working solutions	Volume/reaction										
FVL DIL, vial 4	11 µL										
FVL MD Mix, vial 1	2 µL										
FVL R Mix, vial 2	2 µL										
Total volume	15 µL										
2	<ul style="list-style-type: none"> • Mix gently. • Pipet 15 µL Master Mix into each precooled LightCycler® Capillary. • Verify the identity of the specimen list (Chapter 1, A7, printout) with the respective Sample Cartridge by comparing the barcode numbers (optional). • Add 5 µL of isolated specimen DNA or 5 µL of FVL CT (vial 3, purple cap) as a positive control or 5 µL FVL DIL (vial 4, colorless cap) as a negative control. 										
3	Seal each capillary with a stopper using the LC Capping Tool (Cat. No. 03 357 317 001).										
4	<p>If using the LC Carousel Centrifuge, proceed to step 5.</p> <p>If utilizing an equivalent bench top centrifuge (like the Biofuge 13 from Heraeus Instruments or Kendro Laboratory Products), place each capillary into the respective precooled LightCycler® Centrifuge Adapters and perform the following steps:</p> <ul style="list-style-type: none"> • Place adapters in the benchtop centrifuge • Centrifuge at max. 735 × g for 30 s. • Proceed with step 5. 										
5	Place the capillary containing the negative control in position 1, and the capillary containing the positive control in position 2 of the LightCycler® Carousel.										
6	Starting with position 3, place the capillaries containing the specimen DNA in the LightCycler® Carousel.										
7	Transfer the rotor bucket, including the LightCycler® Carousel, to the LC Carousel Centrifuge 2.0 or LC Carousel Centrifuge equipped with the LC 2.0 Carousel Centrifuge rotor set.										
8	Press Start to commence the LC Carousel Centrifuge run.										
9	After centrifugation, transfer the LightCycler® Carousel to the LightCycler® 2.0 Instrument.										

C. AMPLIFICATION AND DETECTION LightCycler® 2.0 Instrument

Note: Before starting the Macro, log onto an Exor Database (DB) with an audit trail. If logged onto the Exor DB with an audit trail, the word "Traceable" is displayed in the Status bar of the software. For further information on how to login to the software, refer to the LightCycler® 2.0 Instrument Operator's Manual, Software Version 4.05 or 4.1 for *in vitro* diagnostic use.

1. Place the carousel into the LightCycler® 2.0 Instrument.
 2. Make sure that the Factor V Leiden Kit Macro (Cat. No. 04 586 913 001) is installed. This Macro only needs to be installed once when performing the test for the first time.
 3. Start the macro by pushing the Roche button and entering the Cat. No. of the Kit, either manually or by scanning the barcode. Refer to the back of this package insert for the correct kit specific barcode information.
- Note:** "Perform self test" may not be active.
4. The kit wizard will guide you through the entire procedure.
 5. Enter the specimen number in the sample count section and the specimen names on the **Capillary View** screen, according to the actual sample loading scheme. Enter the Kit lot number in the respective field on the **Capillary View** screen.
 6. Click "Start Run" button within the kit wizard.
 7. Once the run is complete, the LightCycler® 2.0 Instrument automatically determines and displays the genotype by performing a melting curve analysis.

Note: The fluorescence signal is measured in the 640 nm channel.

INSTRUMENT CALIBRATION

No calibration is necessary.

QUALITY CONTROL

The negative and positive controls must be included in each PCR run.

Negative control:

The assay result for the Factor V Leiden negative control should always be "negative". If this is not the case, the whole run is flagged as invalid. The entire procedure (specimen preparation, amplification and detection) must be repeated. If the negative control consistently gives a non-negative result, contact your local Roche representative for technical assistance.

Positive control:

The assay result for the Factor V Leiden CT should always be genotyped as "het".

If this is not observed, the run is invalid and the entire procedure (specimen preparation, amplification and detection) must be repeated.

If the positive control consistently fails for the heterozygous genotype, contact your local Roche representative for technical assistance.

Specimen result:

The assay result for the specimen DNA should always exhibit a melting curve profile for the homozygous wild-type, homozygous mutant or heterozygous genotype. If this is not observed, the result for this specimen is invalid, and the entire procedure (specimen preparation, amplification and detection) must be repeated.

LIMITATIONS AND INTERFERENCES

The FVL MD Mix (vial 1, yellow cap) is sequence specific for the human Factor V gene. High concentrations of heparin might interfere with the polymerase chain reaction. Other possible interferences are not known.

EXPECTED VALUES / INTERPRETATION OF RESULTS

1. Ensure that the control results for the run are valid. If the run is invalid, repeat the entire run (specimen preparation, amplification and detection).
2. For a valid run, specimen results are displayed as follows by the genotyping analysis module of the LightCycler® Software:

Displayed result	Interpretation
wt	homozygous wild-type genotype: specimen is negative for the Factor V Leiden mutation (<i>i.e.</i> , no mutant allele present)
het	heterozygous genotype: specimen carries both alleles, one wild-type and one mutant (Factor V Leiden) allele
mut	homozygous mutant genotype: specimen is positive for the Factor V Leiden mutation (<i>i.e.</i> , two mutant alleles are present)
unknown	<ul style="list-style-type: none"> • Genotype cannot be grouped into the pre-defined melting standards. • Repeat the analysis procedure including specimen preparation, amplification and detection. If the result is again "unknown", contact your local Roche representative for technical assistance.
negative	specimen matches the software criteria for a negative control

SPECIFIC PERFORMANCE DATA

Analytical specificity

Using synthetic oligonucleotides as primers and HybPr[®]be probes, it was shown that the LightCycler® Software 4.05 or 4.1 genotyping clearly identifies the Factor V Leiden mutation. However, it does not allow the differentiation of the rare silent mutations A1692C, G1689A and A1696G. Genotyping with the LightCycler® Software 4.05 or 4.1 will classify these mutations as false positive for Factor V Leiden.

By applying sequence analysis, it was shown that the selected synthetic oligonucleotides used as primers specifically amplify a 222 bp fragment of the Factor V gene containing the Factor V Leiden sequence.

Analytical sensitivity

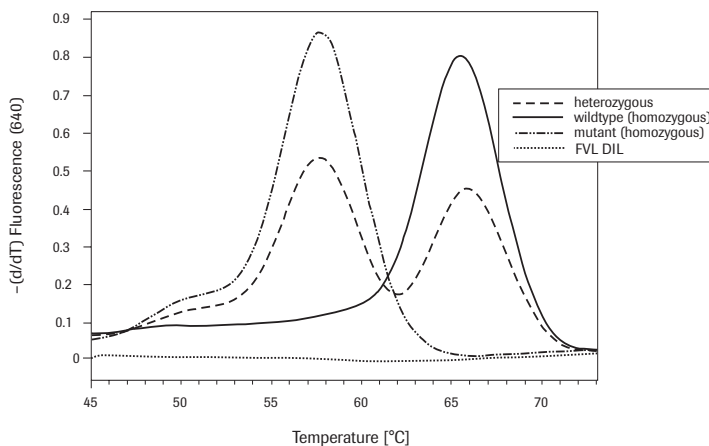
Purified human DNA heterozygous for the Factor V Leiden mutation was diluted in four serial dilution steps. Each dilution was analyzed in six replicates and statistical interpretation of results was performed. A minimum detection level of 202 copies per reaction for the Factor V Leiden Kit was calculated.

Diagnostic sensitivity and specificity

In a prospective study of freshly-collected and repository human DNA specimens, 530 specimens of Caucasian patients with suspected thrombophilia were analyzed using the Factor V Leiden Kit and single or double strand sequence analysis on a MegaBACE 500 Sequencing System using Cimarron Base Caller SW 3.12. Both panels represent entire routine inpatient thrombophilia collectives of a single university hospital laboratory collected over a defined time frame. The level of agreement between the Factor V Leiden Kit and sequence analysis was 99.4% (527/530). Detailed data are shown in the table below.

Table 1: Factor V Leiden Kit versus Sequence Analysis

Sequence analysis	Factor V Leiden Kit			
	wild-type (wt)	heterozygous (het)	mutant (mut)	unknown
wt	440	-	-	2
het	1	82	-	-
mut	-	-	5	-



Note: 41.0% of the human DNA specimens were obtained from patients with suspected venous thromboembolic events in accordance with either or both of the American College of Medical Genetics (ACMG) or College of American Pathologists (CAP) consensus statements (2,4), 37.0% from patients with arterial thrombotic disorders (e.g., stroke), 18.0% from patients referred with suspected thrombophilia for other reasons, and 4.0% for unrecorded reasons. The genotype for two specimens could not be obtained with the Factor V Leiden Kit.

NOTICE TO PURCHASER

The purchase of this product allows the purchaser to use it for amplification and detection of nucleic acid sequences for providing human *in vitro* diagnostics. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

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Manufactured in Germany for
Roche Molecular Systems, Inc., Branchburg, NJ 08876 USA



Roche Diagnostics GmbH, Mannheim, Germany, +49 621 7590

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