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Instructions for Use

imegen™ Factor V dPCR dry

Genotyping of the c.1601G>A; p.Arg534Gln (R506Q) mutation in the *F5* gene (Leiden) by digital PCR

REF IMG-332

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ISO 9001
BUREAU VERITAS Certification
Nº ES063227-1

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Our products are designed for Research Use Only (RUO). Imegen does not offer any other warranty, express or implied, which extend beyond the proper functioning of the components of this kit. Imegen only obligation in respect to the previous guarantees, will be to replace the product or return the purchase price, when desired by the customer, as long as the existence of a defect in the materials or in the manufacturing of its products is proven.

Imegen is not liable for any damage, direct or indirect, resulting in economic loss or resulting from the use of this product by the purchaser or user.

All products sold by the Imegen are subjected to rigorous quality control. The imegen-Factor V dPCR has passed all internal validation tests, ensuring the reliability and reproducibility of each assay.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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Amendments to the Instructions for Use (IFU)	
Version 02	Contents modification

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1. General information

The F5 gene, located on chromosome 1q24.2 region, encodes the coagulation factor V, a plasma glycoprotein that circulates with little or no activity. The active form of Factor V (Factor Va) is essential for the coagulation process and acts as a cofactor in the activation of prothrombin (Factor II).Coagulation factors are a group of related proteins that are essential for normal blood clotting (hemostasis). After an injury, clots protect the body by sealing off damaged blood vessels and preventing further blood loss.

The coagulation system is controlled by several proteins, including a protein called activated protein C (APC). APC normally inactivates coagulation factor V, which slows down the clotting process and prevents clots from growing too large. However, in people with factor V Leiden thrombophilia, coagulation factor V cannot be inactivated normally by APC. As a result, the clotting process remains active longer than usual, increasing the chance of developing abnormal blood clots.

Other factors also increase the risk of developing blood clots in people with factor V Leiden thrombophilia. These factors include increasing age, obesity, injury, surgery, smoking, pregnancy, and the use of oral contraceptives (birth control pills) or hormone replacement therapy. The risk of abnormal clots is also much higher in people who have a combination of the factor V Leiden mutation and another mutation in the F5 gene. Additionally, the risk is increased in people who have the factor V Leiden mutation together with a mutation in another gene involved in the coagulation system.

References

- Rosendaal FR, Reitsma PH. Genetics of venous thrombosis . J Thromb Haemost. 2009;7: 301–304.
- Rosendorff A, Dorfman DM. Activated protein C resistance and factor V Leiden: a review . Arch Pathol Lab Med. 2007;131: 866–71.
- Segal JB, Brotman DJ, Necochea AJ, Emadi A, Samal L, Wilson LM, et al. Predictive Value of Factor V Leiden and Prothrombin G20210A in Adults With Venous Thromboembolism and in Family Members of Those With a Mutation . JAMA. 2009;301: 2472.

2. Intended use

imegen-Factor V dPCR employs a combination of oligonucleotides and fluorescent hydrolysis probes in a qualitative digital PCR diagnostic assay directed to simultaneously amplify and detect the F5 wild type (c.1601G) and the particular mutation in the F5 gene (c.1601A; p.Arg534Gln) that causes factor V Leiden thrombophilia, a deficit in the enzyme activity. This deficiency has been associated with hereditary thrombophilia, Budd-Chiari syndrome, stroke and slightly increased susceptibility to recurrent pregnancy loss.

This genetic analysis enables the user to detect the presence or absence of such genotypes in one single multiplexed reaction as each target is labelled a different fluorophore. The presence of the wild type (c.1601G) is included as a control of the DNA quality and integrity.

The assay analyses the genotype in the germline, thus the optimal sample type required for this analysis is genomic DNA (gDNA). Each reaction uses a total of 30 ng of gDNA.

imegen-Factor V dPCR has been designed for Research Use Only and it is directed to professionals from the molecular biology sector.

3. Technical characteristics

imegen-Factor V dPCR consists of an end-point PCR assay aimed at quantifying by digital PCR the copy number of the targets. The assay has been validated with genomic DNA (gDNA) extracted from peripheral blood from diagnostic samples that were previously genotyped using a different technique, as well as certified synthetic vectors (GenScript) containing the sequences of interest. The thorough validation provides a robust and specific diagnostic method.

For the use of imegen-Factor V dPCR dry kit is necessary a Digital PCR system with the fluorescence channels:FAM™ and VIC® (HEX).

The type of sample required for this analysis is genomic DNA extracted from peripheral blood, a total quantity of 60 ng will be necessary. The limit of detection (LOD) is established to be 10 ng of genomic DNA.

This product complies with the quality requirements established by ISO 9001, both in its validation and manufacturing process as well as in the materials used.

4. Warnings and precautionary statements

1. Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.

2. Do not pipette by mouth.

3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

4. You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.

5. Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.

6. In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.

7. The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to imegen.

8. This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.

9. Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.

10. This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.

11. The manufacturer is not responsible for the malfunction of the assay when one or more reagents included in the kit are replaced by other reagents not supplied by Imegen.

12. The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Imegen, considering them equivalent to those provided in the Kit.

5. Contents and storage conditions

The kit contains reagents to perform 48 dPCR determinations:

- Factor V Master Mix: Lyophilised master mix containing the oligonucleotides and hydrolysis probes for the multiplexed amplification of the wild type and the mutated genotype associated to Leiden. F5 wild type and F5 mutated genotype are labelled with fluorophores VIC® and FAM™, respectively.
- Factor V Control: Aqueous positive control is heterozygous for the G-to-A transition at position c.1601 of the Leiden gene (c.1601G>A; p.Arg534Gln).

Reagents	Quantity	Storage
Factor V Master Mix	4 x 12 rxn	4 °C
Factor V Control	60 µL	4 °C

Table 1. imegen-Factor V dPCR dry contents and storage temperature.

6. Equipment and materials required but not supplied

Equipment:

- Micropipette (10 µL, 20 µL and 200 µL)

QuantStudio 3D Digital PCR (ThermoFisher Scientific)
- QuantStudio 3D Digital PCR (Chip loader)
- ProFlex™ 2x Flat PCR System (dPCR thermalcycler)
- QuantStudio 3D Digital PCR instrument (Chip reader)

Droplet Digital PCR (Bio-Rad)
- QX200TM Droplet DigitalTM PCR system o QX100TM Droplet DigitalTM PCR system
- PX1™ PCR Plate Sealer
- C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module

Reagents:

- Nuclease-free water

QuantStudio 3D Digital PCR (ThermoFisher Scientific)
- QuantStudio 3D Digital PCR Master Mix v.2
- Absolute ethanol

Droplet Digital PCR (Bio-Rad)
- ddPCRTM Supermix for probes (No dUTP)
- Droplet generation oil for probes

Materials:

- Sterile tubes 0.2 mL and 1.5 mL
- Pipette filter tips (10 µL, 20 µL and 200 µL)
- Dust-free gloves

QuantStudio 3D Digital PCR (ThermoFisher Scientific)
- QuantStudio® 3D Digital PCR 20K Chip Kit v2 (12 pack) (Ref.A26316)
- Dust-free tissues

Droplet Digital PCR (Bio-Rad)
- Droplet Generator Cartridges and Gaskets (Ref.1864007)
- ddPCR 96-well plates (Ref.12001925)
- Pierceable PCR plate heat seal (Ref.1814040)

6.1 Related kits

Related haematology assays include:

Name of the kit	Reference
imegen-Factor II dPCR dry (Digital PCR)	IMG-331
imegen-Factor II (Real-time PCR)	IMG-214
imegen-HFE (Real-time PCR)	IMG-218
imegen-Cambridge II (Real-time PCR)	IMG-199
imegen-Factor XII (Real-time PCR)	IMG-215
imegen-MTHFR (Real-time PCR)	IMG-212
imegen-MTHFRII (Real-time PCR)	IMG-216

Table 2. Haematology kits using real-time PCR or digital PCR.

7. Assay protocol

7.1 Preparation of the PCR reagents

The first step before using the kit consists of rehydrating the Factor V master mix:

Reagents	Volume of nuclease-free water
Factor V Master Mix	20 µL of water/vial

Table 2. Rehydration of lyophilised reagents.

For optimal resuspension of each component, we recommend to mix thoroughly the contents and spinning the tubes before letting them to stand for an hour at 4°C. If the reagents will not be used after rehydration, we recommend storage at -20 °C.

The positive control is provided in aqueous phase and long-term storage at -20°C is recommended.

7.2 Set up of PCR assay

The assay must be include the follow reactions:

- Unknown sample
- Positive control (Factor V Control)
- Recommended: Negative control reaction (this reaction contain nuclease-free water, to guarantee the absence of contamination in the process).

To set up the digital PCR reactions following the protocol below:

1. Thaw all the reagents needed for the analysis including,
    a. Genomic DNA samples. Diluted at optimal concentration of 10 ng/µl
    b. Factor V Master Mix (Rehydrated)
    c. Factor V Control
    d. Nuclease-free water for the negative controls (no template controls, NTC)
    e. Digital PCR Master Mix (not provided)
2. Vortex and spin each reagent to mix thoroughly and keep on ice. The PCR will vary depending on the digital PCR system utilised:

QuantStudio® 3D Digital PCR system (ThermoFisher Scientific)

3. Add the following reagents into a fresh 1.5 mL tube:

Reagents	Amount per reaction
Factor V Master Mix	1.5 µL
QuantStudio 3D Digital PCR Master Mix v.2	7.5 µL

Table 3. Amount of reagents required to perform one analysis with the QuantStudio 3D dPCR

Note: To estimate the total amount of reagents, we recommend making the calculations taking into account the number of samples to be simultaneously analysed, and to add up an extra 10% the volume of each reagent.

4. Mix the PCR Master Mix tube by pipetting up and down carefully not to create bubbles (Do not vortex), and dispense 9 µL into the corresponding 0.2 mL tubes.

5. Add 6 µL of sample DNA at 5 ng/µL, or of nuclease-free water (negative control) into the corresponding tubes.

6. Load 14.5 µL of the PCR reaction on the QuantStudio 3D Digital PCR Chip Loader, according to the manufacturer's instructions for loading the chip.

Droplet Digital™ PCR system (BIO-RAD)

3. Add the following reagents into a fresh 1.5 mL tube:

Reagents	Amount per reaction
Factor V Master Mix	1.5 µL
ddPCR™ Supermix for probes (No dUTP)	10 µL
Nuclease free water	2.5 µL

Table 4. Amount of reagents required to perform one analysis with the Droplet Digital PCR

Note: To estimate the total amount of reagents, we recommend to make the calculations taking into account the number of samples to be simultaneously analysed, and to add up an extra 10% the volume of each reagent.

4. Mix the PCR Master Mix tube by pipetting up and down carefully not to create bubbles (Do not vortex), and dispense 14 µL the specified volumes into a fresh 96-well plate.

   Note: If any of the wells in the columns of the plate having samples are empty, add 20 µl of buffer control or nuclease-free water.

5. Add 6 µL of sample DNA at 5 ng/µL, or of nuclease-free water (negative control) into the corresponding wells.

6. Load 20 µL of the PCR reaction with the multichannel pipette into the corresponding wells of the loading cartridge, according to the manufacturer's instructions.

7.3 Setup of the digital PCR programme

QuantStudio® 3D Digital PCR system (ThermoFisher Scientific)

- Chip loading

  To load, assemble and seal properly the dPCR chips (fungible not included) follow the manufacturer's instructions. Please download the user guide: MAN0007720 QuantStudio ™ 3D Digital PCR System User Guide available on their website www.thermofisher.com and follow the instructions on Chapter 3.

- Setup of the dPCR programme

  In order to place the chips on the thermal cycler: ProFlex™ 2x Flat PCR System, please download the user guide: MAN0007720 QuantStudio™ 3D Digital PCR System User Guide on the website and follow the instructions on Chapter 4.

  - Optimal dPCR programme:

Fields	Step 1 Enzymatic activation	Step 2 PCR		Step 3	
Number of Cycles	1 Initial Cycle (Denat.)	40 cycles		1 Final Cycle	Conservation
		Annealing & Extension	Denat.		
Temperature	96°C	56°C	98°C	60°C	20°C
Time	10 minutes	2 minutes	30 seconds	2 minutes	∞

Table 5. Optimal dPCR programme for the QuantStudio 3D dPCR System

- Reading the chips and obtaining results

  Once the PCR programme is finished, follow the instruction on Chapter 5 of the user guide: MAN0007720 QuantStudio™ 3D Digital PCR System User Guide to retrieve the files resulting from the chips reading.

Droplet Digital™ PCR system (BIO-RAD)

- Load amplification reactions in the cartridge

  To prepare the sample-oil emulsions and transfer the emulsions into the cartridges (fungible not included) follow the manufacturer's instructions for either one of the QX200™ Droplet Digital™ PCR system or QX100TM Droplet DigitalTM PCR system (BIO-RAD). Please download the Droplet Digital™ PCR Applications Guide available on the web BioRad website www.bio-rad.com and follow the instructions on Chapter 2, Section ddPCR Experimental Workflow> Droplet Generation.

- dPCR program setup

  Place the 96-well ddPCR plate into the C1000 Touch ™ Thermal Cycler with 96-Deep Well Reaction Module and set up the following PCR programme:

  - Optimal PCR programme:

Fields	Step 1 Enzymatic activation	Step 2 PCR		Step 3	
Number of Cycles	1 Initial Cycle (Denat.)	40 cycles		1 Final Cycle	Conservation
		Denat.	Annealing & Extension		
Temperature	96°C	94°C	60°C	98°C	20°C
Time	10 minutes	30 seconds	1 minute	10 minutes	∞

Table 6. Optimal dPCR programme for the Bio-Rad dPCR platform.

- Reading the plate fluorescence and obtaining results

  Once the PCR program is finished, follow the instructions on Chapter 2 and, specifically the sections Setting Up an Experiment in Quantasoft™ Software and Droplet Reading, of Droplet Digital™ PCR Applications Guide available on the webpage www.bio-rad.com to obtain the files resulting from the plate reading. As type of Experiment, select the RED option: rare target sequence detection (rare event detection).

8. Results analysis

For the correct interpretation of the results, it is important to know the fluorophore used to label each one of the two molecular targets.

Hydrolysis Probe	Fluorophore
***F5* Wild type (c.1601G)**	VIC®
***F5* Mutated (c.1601A)**	FAM™

Table 7. Details about the hydrolysis probes.

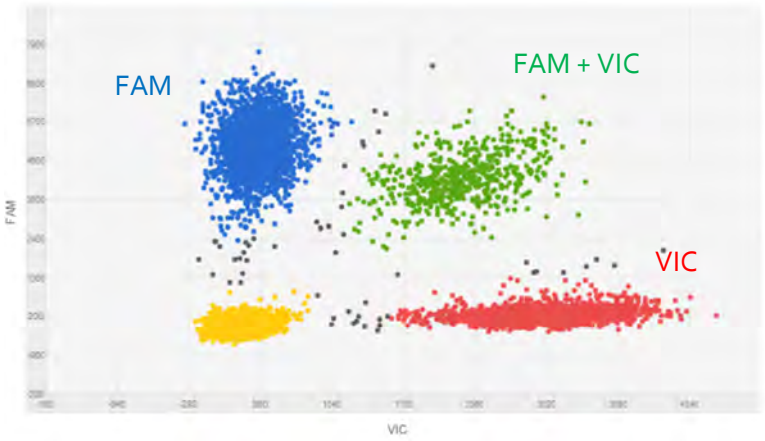

Figure 1. Graphic representation of a heterozygous sample for the G-to-A transition at position c.1601 of the *F5* Leiden gene (c.1601G>A).

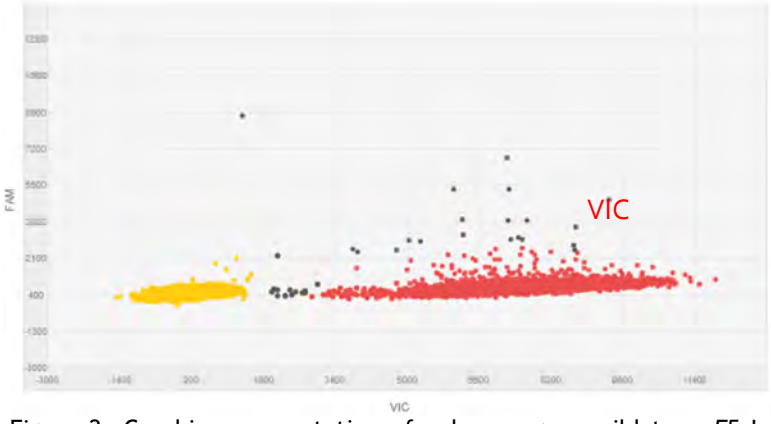

Figure 2. Graphic representation of a homozygous wild type *F5* Leiden gene (c.1601G).

QuantStudio® 3D Digital PCR system (ThermoFisher Scientific)

The dPCR results are analysed using the QuantStudio™ 3D AnalysisSuite™ online software available from the ThermoFisher Scientific website, www.thermofisher.com. In order to access it, the user will firstly have to register prior to start using the application. Please, follow the instructions on Chapter 5 of the MAN0007720 QuantStudio™ 3D Digital PCR System User Guide, for more details. An additional section on Troubleshooting is available on the User Guide.

In addition, the following guidelines should be taken into account for a correct analysis of the results:

- Negative Controls (NTC). Confirm that there is no amplification signals for neither of the targets (FAM and VIC). If amplification is detected, a repeated analysis should be performing to rule out the possibility of an accidental contamination.
- It is recommended to review and, if needed, manually edit the results obtained for each analysed chip by accessing the "Review Data" tab.
- For the analysis of the results, access the "Review Data" to visualise the results or "Export" tab to export the copies/µl (FAM) of mutated genotype and the copies/µL (VIC) for the wild type genotype to determine the allelic load in the sample:

$$\%\,\boldsymbol{Zygosity} = \frac{\frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{FAM})}{\frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{VIC}) + \frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{FAM})}\boldsymbol{x}\,\mathbf{100}$$

  - If both alleles of a diploid organism are the same, the organism is homozygous at that locus. If they are different, the organism is heterozygous at that locus:
    - 50% Zygosity. F5 Heterozygous (c.1601G>A)
    - 100% Zygosity. F5 Homozygous wild type genotype (c.1601G)
    - 0% Zygosity. F5 Homozygous mutant genotype (c.1601A)

Droplet Digital™ PCR system (Bio-Rad)

The QuantaSoft™ Software from Bio-Rad is used for the analysis of the results. To facilitate the interpretation of the data, follow the instructions on Chapter 2, specifically the details included on the Data analysis of the Droplet Digital™ PCR Applications Guide, available on the BioRad website www.bio-rad.com. An additional section on Troubleshooting is available on the User Guide.

- It is recommended to review and, if needed, manually edit the results obtained for each sample.
- For the analysis of the results, access the "Analyze>Concentration" tab from the analysis software to calculate the percentage of chimerism in the sample:

$$\%\ \boldsymbol{Zygosity} = \frac{\frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{FAM})}{\frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{VIC}) + \frac{\boldsymbol{Copies}}{\mu\boldsymbol{L}}(\boldsymbol{FAM})}\boldsymbol{x}\ \mathbf{100}$$

  - If both alleles of a diploid organism are the same, the organism is homozygous at that locus. If they are different, the organism is heterozygous at that locus:
    - 50% Zygosity. F5 Heterozygous (c.1601G>A)
    - 100% Zygosity. F5 Homozygous wild type genotype (c.1601G)
    - 0% Zygosity. F5 Homozygous mutant genotype (c.1601A)

9. Troubleshooting

The table below represents the results that could be obtained using the positive and negative controls and the cDNA samples. In case an unexpected result is obtained, the interpretation of the result and the cause most likely reason for such result is given in the table below.

Control	F5 Mutated	F5 Wild type	Result / Interpretation
Positive Control	+	+	Expected result
	-	-	Fail in the PCR setup [1]
DNA sample	-	+	Expected result
	+	+	
	+	-	
	-	-	Fail to amplify the DNA sample [2]
Negative Control (NTC)	-	-	Expected Result
	+	+	Contamination with human DNA or with the positive control [3]

Table 8. Interpretation of the possible results obtained using imegen-Factor V dPCR dry.

[1] Fail in the PCR setup: An error in the amplification might be due to a technical issue during the configuration of the PCR system. Check the amplification programme and the setup of the fluorescence detection.

[2] Fail to amplify the DNA sample: An error to amplify the F5 gene in the gDNA sample might suggest the quantity or the quality of the gDNA sample is compromised. In this situation, a second analysis would be recommended before an interpretation of the results is made.

[3] Contamination with human DNA: PCR contamination might be caused by an inappropriate handling of the sample, the use of contaminated reagents or caused by an environmental contamination. To solve this issue, a thorough cleanse of the laboratory where the PCRs are prepared, including the equipment and material used is recommended. If necessary, use fresh aliquots of the PCR reagents.

10. Limitations

10.1 Equipment

Imegen-Factor V dPCR dry has been validated using the following digital PCR systems:

- QuantStudio® 3D Digital PCR system (ThermoFisher Scientific)

If a digital PCR system different from the systems described in this section is going to be used for the quantification of molecular chimerisms with this kit, it is possible that the PCR programme might need to be readjusted. In this case, please contact our Technical Support Team for more details.

10.2 Reagents

imegen-Factor V dPCR dry has been validated using the reagents included in the kit and the reagents recommended by the supplier of the dPCR systems, as indicated in Section 6 (Equipment and Materials needed but not included in the kit).

It is highly recommended to use the dPCR reagents provided by the thermal cycler supplier. Please, contact our Technical Support Team if you request any further information.

10.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied as specified on Section 5 (Contents and Storage Conditions) from the reception of the kit until the expiry date assigned to each production batch.