



# Instructions for use

## Imegen<sup>®</sup> Factor II dPCR

Ref. IMG-331



Genotyping of mutation c.\*97G> A (20210G> A) in gene F2 (prothrombin) by digital PCR

Manufactured by:

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Code: HIC-PT-KIT 03-F-03 V.01

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**Our products are intended solely for research use.** Health in Code, S.L. provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. The sole obligation of Health in Code, S.L. in relation to the aforementioned guarantees shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship prove to be defective. Health in Code, S.L. is not liable for any cost or expense, either direct or indirect, or any damage or harm incurred by the customer or user as a result of use of the product by the buyer or user.

All Health in Code, S.L. products undergo strict quality control. The **Imegen® Factor II dPCR** kit has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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Modifications to the Instructions for Use (IUF)		
Version 04	FEB 2023.	Correction about assigning the mutant homozygote and the native homozygote in section 8.
Version 03	DEC 2022	Change of manufacturer's address to: Health in Code, S.L., Calle de la Travesía s/n, 15E Base 5, Valencia, 46024, Spain
Version 02	SEP 2022	Change of manufacturer's identification from Imegen to Health in Code, S.L.
Version 01	JUL 2019	Content review

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# 01 General information

The *F2* gene, located in region 11p11.2 of the chromosome, provides instructions for producing a protein known as prothrombin (also referred to as coagulation factor II). Coagulation factors are a group of related proteins that are essential for normal blood coagulation (hemostasis). After an injury, blood clotting protects the body by sealing damaged blood vessels, thus preventing further blood loss.

Prothrombin is synthesized in the liver as an active zymogen. The protein circulates in the bloodstream in its inactive form until an injury damages blood vessels. As a response to said injury, prothrombin turns into its active form, thrombin. Thrombin then converts a protein known as fibrinogen into fibrin, the main protein that forms blood clots, stimulates platelet aggregation, and activates coagulation factors V, VIII, and XIII.

Thrombin is also believed to be involved in cell growth and division (proliferation), tissue repair, new blood vessel formation (angiogenesis), and inhibition of coagulation by activating protein C.

## References

- > *Kujovich JL. Prothrombin-Related Thrombophilia [Internet]. GeneReviews®. University of Washington, Seattle; 1993.*
- > *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.*
- > *Rosendaal FR, Reitsma PH. Genetics of venous thrombosis. J Thromb Haemost. 2009;7: 301–304.*

## 02 Intended use

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**Imegen® Factor II dPCR** uses a combination of oligonucleotides and fluorescent hydrolysis probes in a qualitative diagnosis assay by digital PCR aimed at simultaneously amplifying and detecting the native *F2* genotype (20210G) and the most common mutant phenotype (20210A) associated with prothrombin-related thrombophilia.

This genetic test allows the user to detect the presence or absence of these genotypes with a single multiplexed reaction, as each target is labeled with a different fluorophore. The presence of the native genotype (20210G) is included as a control for DNA quality and integrity.

The assay studies the germline genotype; therefore, the optimal sample type for this analysis is genomic DNA (gDNA). Each reaction uses a total gDNA amount of 30 ng.

**Imegen® Factor II dPCR** is intended solely for research use and is aimed at professionals working in molecular biology.

## 03 Technical characteristics

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**Imegen® Factor II dPCR** consists of an endpoint PCR assay aimed at quantifying the copy number of the targets by digital PCR. The assay has been validated using genomic DNA from peripheral blood obtained from diagnostic samples that had previously been genotyped using a different technique, as well as reference samples from the *Coriell Research Institute*. The complete validation gives a robust and specific diagnosis method.

The use of the **Imegen® Factor II dPCR** dry kit requires a digital PCR system with fluorescence channels FAM™ and VIC™ (HEX).

The sample type required for this test is genomic DNA from peripheral blood, and a total amount of 60 ng is needed. The limit of detection has been determined at 10 ng of genomic DNA.

## 04 Safety warnings and precautions

- ◇ It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- ◇ Do not mouth-pipette.
- ◇ Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- ◇ Any cuts, abrasions, and other skin injuries must be properly protected.
- ◇ Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- ◇ In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- ◇ Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- ◇ This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- ◇ The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- ◇ This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory conduct an internal validation when the kit is to be used for the first time.
- ◇ The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code, S.L.
- ◇ The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code, S.L. but are considered by the user equivalent to those provided in the kit.

## 05 Content and storage conditions of the kit

The kit contains the necessary reagents to perform 48 dPCR determinations:

- **Factor II Master Mix:** freeze-dried master mix containing the necessary oligonucleotides and hydrolysis probes for the multiplexed amplification of the native genotype and the prothrombin-associated mutated genotype. The native and mutated *F2* phenotypes are labeled using VIC™ and FAM™ fluorophores, respectively.
- **Factor II Control:** the aqueous-phase positive control is heterozygous for the G-to-A transition at position 20210 of the prothrombin gene (20210G>A).

Reagents	Quantity	Conservation
<b>Factor II Master Mix</b>	4 x 12 rxn	4 °C
<b>Factor II Control</b>	60 µl	4 °C

Table 1. Content and storage temperature.



## 06

# Equipment, reagents and materials not included in the kit

**Equipment:**

- Micropipettes (10  $\mu$ L, 20  $\mu$ L, and 200  $\mu$ L)
- ↳ **QuantStudio 3D Digital PCR (Thermo Fisher Scientific)**
  - *QuantStudio 3D Digital PCR* (chip loader)
  - *ProFlex™ 2x Flat PCR System* (dPCR thermal cycler)
  - *QuantStudio 3D Digital PCR Instrument* (chip reader)
- ↳ **Droplet Digital PCR (Bio-Rad)**
  - *QX200™ Droplet Digital™ PCR system* or *QX100™ Droplet Digital™ PCR system*
  - *PX1™ PCR Plate Sealer*
  - *C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module*

**Reagents:**

- Nuclease-free water
- ↳ **QuantStudio 3D Digital PCR (Thermo Fisher Scientific)**
  - *QuantStudio 3D Digital PCR Master Mix v.2*
  - Absolute ethanol
- ↳ **Droplet Digital PCR (Bio-Rad)**
  - *ddPCR™ Supermix* for probes (no dUTP)
  - Droplet generation oil for probes

**Materials:**

- Sterile 0.2 and 1.5 ml tubes
- Pipette filter tips. (10  $\mu$ L, 20  $\mu$ L, and 200  $\mu$ L)
- Gloves
- ↳ **QuantStudio 3D Digital PCR (Thermo Fisher Scientific)**
  - *QuantStudio® 3D Digital PCR 20K Chip Kit v2* (12-unit pack) (Ref. A26316)
  - Powder-free towels

### ↳ Droplet Digital PCR (Bio-Rad)

- Droplet generator cartridges and gaskets (Ref. 1864007)
- 96-well plates for ddPCR (Ref. 12001925)
- Pierceable PCR plate heat seal (Ref. 1814040)

## Complementary kits

Other related hematology assays include:

Kit name	Reference
<b>Imegen® Factor V dPCR</b> (digital PCR)	IMG-332
<b>Imegen® Factor V</b> (real-time PCR)	IMG-217
<b>Imegen® HFE</b> (real-time PCR)	IMG-218
<b>Imegen® Cambridge II</b> (real-time PCR)	IMG-199
<b>Imegen® Factor XII</b> (real-time PCR)	IMG-215
<b>Imegen® MTHFR</b> (real-time PCR)	IMG-212
<b>Imegen® MTHFR II</b> (real-time PCR)	IMG-216

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

# 07 Assay protocol

## 07.1 | Preparation of reagents for PCR

The first step before using the kit consists of rehydrating the *Factor II Master Mix*:

Reagents	Nuclease-free water volume
<i>Factor II Master Mix</i>	20 $\mu$ L of water/vial

Table 3. Rehydration of freeze-dried reagents.

For optimal resuspension of each component, we recommend thoroughly mixing the content and spinning the tubes before leaving to rest for one hour at 4 °C. If the reagents are not to be reutilized after rehydration, we recommend storing at -20 °C.

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

## 07.2 | Settings for the PCR assay

The assay must include the following reactions:

- ◇ Unknown sample.
- ◇ Positive control (*Factor II Control*).
- ◇ Recommended: negative control reaction (a reaction that contains nuclease-free water to confirm the absence of contamination during the process).

Configure the digital PCR reactions following the protocol below:

- 01 Thaw all the necessary reagents to perform the analysis, including:
  - ◇ Genomic DNA samples diluted to the optimal concentration (10 ng/ $\mu$ L).
  - ◇ *Factor II Master Mix* (rehydrated).
  - ◇ *Factor II Control*.
  - ◇ Nuclease-free water for negative controls (no template controls, NTC).
  - ◇ *Digital PCR Master Mix* (not provided).
- 02 Vortex and spin each reagent to mix thoroughly, then keep on ice. The PCR will vary depending on the digital PCR system used:

### ➤ QuantStudio® 3D Digital PCR system (Thermo Fisher Scientific)

03 Add the following reagents to a new 1.5 mL tube:

Reagents	Volume per reaction
<b>Factor II Master Mix</b>	1.5 µl
<b>QuantStudio 3D Digital PCR Master Mix v.2</b>	7.5 µl

Table 4. Volume of reagents needed in order to carry out the test using the QuantStudio 3D dPCR.

**NOTE:** To estimate the total amount of reagents, we recommend performing the calculations based on the number of samples that will be analyzed simultaneously, then adding an additional 10% to the volume of each reagent.

- 04 Mix the *PCR Master Mix* by pipetting up and down carefully so as not to generate bubbles (do not shake) and dispense 9 µL into the corresponding 0.2 mL vials.
- 05 Add 6 µL of the DNA sample at 10 ng/µL or of nuclease-free water (negative control) to the corresponding vials.
- 06 Load 14.5 µL of the PCR reaction into the *QuantStudio 3D Digital PCR Chip Loader* to load the chip, following the manufacturer's instructions.

### ➤ Droplet Digital™ PCR system (Bio-Rad)

03 Add the following reagents to a new 1.5 mL tube:

Reagents	Volume per reaction
<b>Factor II Master Mix</b>	1.5 µl
<b>ddPCR™ Supermix for probes (No dUTP)</b>	10 µl
<b>Nuclease-free water</b>	2.5 µl

Table 5. Volume of reagents needed in order to carry out the test using the Droplet Digital PCR.

**NOTE:** To estimate the total amount of reagents, we recommend performing the calculations based on the number of samples that will be analyzed simultaneously, then adding an additional 10% to the volume of each reagent.

- 04 Mix the PCR master mix vial by pipetting up and down carefully so as not to generate bubbles (do not shake) and dispense 14 µL of the specified volumes into a new 96-well plate.

**NOTE:** If there are any empty wells in the plate columns containing samples, add 20 µL of control buffer or nuclease-free water.

- 05 Add 6 µL of the DNA sample at 10 ng/µL or of nuclease-free water (negative control) to the corresponding vials.
- 06 Load 20 µL of the PCR solution with a multi-channel pipette in the corresponding loading cartridge, according to the manufacturer's recommendations.

## 07.3 | Settings for the digital PCR program

### ➤ QuantStudio® 3D Digital PCR system (Thermo Fisher Scientific)

#### ↳ Loading the chip:

To successfully load, assemble, and seal the dPCR chips (consumables not provided), follow the manufacturer's instructions. Please download the MAN0007720 *QuantStudio™ 3D Digital PCR System User Guide*, available at the [www.thermofisher.com](http://www.thermofisher.com) website, and follow the instructions in Section 3.

#### ↳ Settings for the dPCR program:

To place the chips into the thermal cycler *ProFlex™ 2x Flat PCR System*, please download the MAN0007720 *QuantStudio™ 3D Digital PCR System User Guide* from the website and follow the instructions in Section 4.

#### ◇ Optimal program:

Fields	Step 1 Enzymatic activation	Step 2 PCR		Step 3	
No. of cycles	1 initial cycle (denaturation)	40 cycles		1 final cycle	Conservation
		Annealing and extension	Denaturation		
Temperature	96 °C	56 °C	98 °C	60 °C	20 °C
Time	10 minutes	2 minutes	30 seconds	2 minutes	∞

Table 6. Optimal dPCR program for the QuantStudio 3D dPCR system.

#### ↳ Analysis of results:

Once the PCR program has been completed, please follow the instructions in Section 5 of the MAN0007720 *QuantStudio™ 3D Digital PCR System User Guide* to retrieve the chip read files.

### ➤ Droplet Digital™ PCR system (Bio–Rad)

#### ↳ Loading the amplification reactions onto the cartridge:

To prepare the oil sample emulsions and transfer them to the cartridges (consumables not provided), follow the manufacturer's instructions for the *PCR QX200™ Droplet Digital™* or *QX100™ Droplet Digital™ PCR* (Bio–Rad) systems. Download the *Droplet Digital™ Applications Guide*, available at the Bio–Rad website [www.bio-rad.com](http://www.bio-rad.com), and follow the instructions in Section 2, *ddPCR Experimental workflow > Droplet generation*.

#### ↳ Settings for the dPCR program:

Place the 96–well dPCR plate into the *C1000 Touch™* thermal cycler with the 96–deep well reaction module and set the following PCR program.

◇ Optimal program:

Fields	Step 1 Enzymatic activation	Step 2 PCR		Step 3	
<b>No. of cycles</b>	1 initial cycle (denaturation)	40 cycles		1 final cycle	Conservation
		Denaturation	Annealing and extension		
<b>Temperature</b>	96 °C	94 °C	60 °C	98 °C	20 °C
<b>Time</b>	10 minutes	30 seconds	1 minute	10 minutes	∞

Table 7. Optimal dPCR program for the Bio-Rad dPCR platform.

↳ Fluorescent plate reading and generation of results:

Once the PCR program is completed, please follow the instructions in Section 2, specifically in sections *Setting up an experiment using the Quantasoft™ software* and *Droplet reading*, of the *Droplet Digital™ PCR Applications Guide*, available at the [www.bio-rad.com](http://www.bio-rad.com) website, to generate the *Plate reading result files*. As experiment type, select the option RED: *rare target sequence detection (detection of rare events)*.

# 08 Analysis of results

To correctly interpret the results, it is important to know the fluorophore used to label each of the two molecular targets:

Hydrolysis probes	Fluorophore
Native F2 genotype (20210G)	VIC™
Mutated F2 genotype (20210A)	FAM™

Table 7. Details about hydrolysis probes.

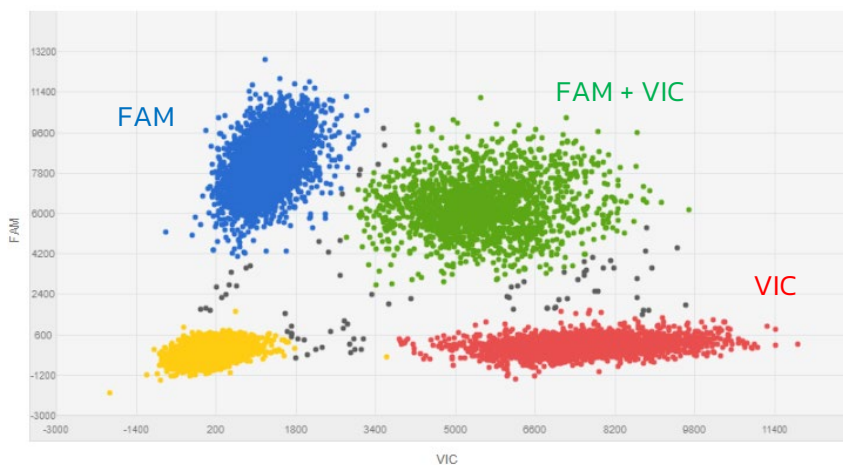


Figure 1. Plot for a heterozygous sample for the G-to-A transition at position 20210 of the prothrombin F2 gene (20210G>A).

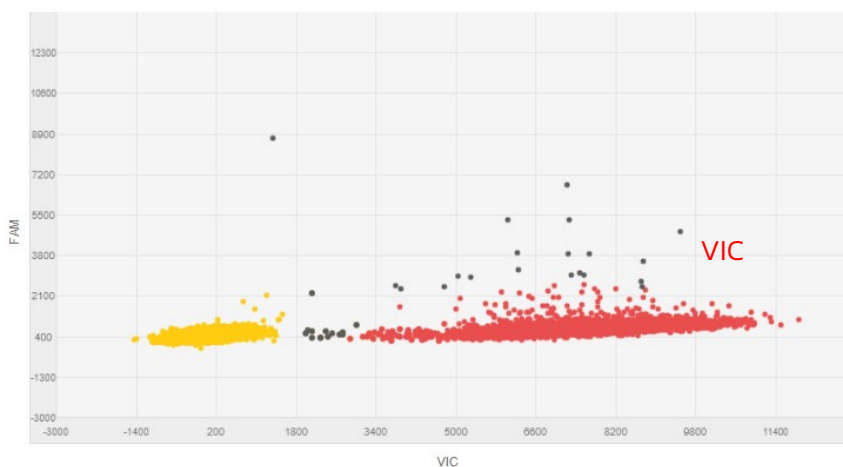


Figure 2. Plot for a homozygous sample for the native prothrombin F2 genotype (20210G)

### ➤ QuantStudio® 3D Digital PCR system (Thermo Fisher Scientific)

The dPCR results are analyzed using the online software *QuantStudio™ 3D AnalysisSuite™*, available at the ThermoFisher Scientific website, [www.thermofisher.com](http://www.thermofisher.com). The user needs to register before accessing the software and using the application. For additional details, follow the instructions in Section 5 of the MAN0007720 *QuantStudio™ 3D Digital PCR System User Guide*. An additional section on troubleshooting is available in the User Guide.

Moreover, the recommendations below should be followed to ensure adequate analysis of the results:

- ◇ **Negative controls.** Verify the absence of amplification signals for any target (FAM and VIC). If amplification is observed, the process should be repeated to rule out the possibility of accidental contamination.
- ◇ It is recommended to review and, if appropriate, manually edit the results obtained for each analyzed chip by clicking on the “Review data” tab.
- ◇ To analyze the results, click on the “Review data” tab to view the results or on the “Export” tab to export the copies/μL (FAM) of the mutated genotype and the copies/μL (VIC) of the native genotype to determine the allelic load of the sample:

$$\% \text{Zygoty} = \frac{\frac{\text{Copies}}{\mu\text{l}} (\text{FAM})}{\frac{\text{Copies}}{\mu\text{l}} (\text{VIC}) + \frac{\text{Copies}}{\mu\text{l}} (\text{FAM})} \times 100$$

- ▾ If both alleles of a diploid organism are the same, the organism is homozygous for that locus. If they are different, the organism is heterozygous for that locus.
  - 50% zygoty. *F2* heterozygote (20210G>A)
  - 100% zygoty. *F2* homozygote for the mutant genotype (20210A)
  - 0% zygoty. *F2* heterozygote for the native genotype (20210G)

### ➤ Droplet Digital™ PCR system (Bio–Rad)

The QuantaSoft™ software by Bio–Rad by is used to analyze the results. To facilitate data interpretation, follow the instructions in Section 2, specifically the details included in section *Data Analysis*, of the *Droplet Digital™ PCR Applications Guide*, available at the [www.bio-rad.com](http://www.bio-rad.com) website. An additional section on troubleshooting is available in the User Guide.

- ◇ It is recommended to review and, if appropriate, manually edit the results obtained for each sample.
- ◇ To analyze the results, click on the tab “Analyze> Concentration” from the analysis software to calculate the chimerism percentage in the sample:

$$\% \text{Zygoty} = \frac{\frac{\text{Copies}}{\mu\text{l}} (\text{FAM})}{\frac{\text{Copies}}{\mu\text{l}} (\text{VIC}) + \frac{\text{Copies}}{\mu\text{l}} (\text{FAM})} \times 100$$



- ↳ If both alleles of a diploid organism are the same, the organism is homozygous for that locus. If they are different, the organism is heterozygous for that locus.
  - 50% zygoty. *F2* heterozygote (20210G>A)
  - 100% zygoty. *F2* homozygote for the mutant genotype (20210A)
  - 0% zygoty. *F2* heterozygote for the native genotype (20210G)

## 09 Troubleshooting

The following table shows the possible results obtained using positive controls, negative controls, and cDNA samples. If an unexpected result is obtained, the interpretation of the result and its most likely reason are given in the following table:

Control	Mutated <i>F2</i>	<i>F2</i> Native	Result/Interpretation
Positive control	+	+	Expected result
	-	-	Incorrect PCR settings <sup>1</sup>
DNA sample	-	+	Expected result
	+	+	
	+	-	
	-	-	DNA sample failed to amplify <sup>2</sup>
Negative control	-	-	Expected result
	+	+	Sample contamination by human DNA <sup>3</sup>

Table 8. Interpretation of possible results obtained using the Imegen® Factor II dPCR dry kit.

(1) **Incorrect PCR settings:** an amplification error may be due to a technical issue during PCR configuration. Make sure the amplification program and fluorescence detection settings are correct.

(2) **DNA sample failed to amplify:** Failure to amplify the *F2* gene in the gDNA sample might suggest that the quantity or quality of the gDNA sample is compromised. In this case, it is advised to perform a new test before interpreting the results.

(3) **Sample contamination by human DNA:** PCR contamination could be caused by improper sample handling, the use of contaminated reagents, or environmental contamination. To fix this issue, deep cleaning of the laboratory where PCRs are prepared is advised, including the equipment and material used. If necessary, use fresh aliquots of the PCR reagents.

# 10 Limitations

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## 10.1 | Equipment

Imegen® Factor II dPCR has been validated using the following digital PCR systems:

- + *QuantStudio® 3D Digital PCR system (Thermo Fisher Scientific)*

Should a digital PCR system different from the ones described in this section be used to quantify molecular chimerisms using this kit, the PCR program may need to be adjusted. In that case, please contact our technical support team for more details.

## 10.2 | Reagents

Imegen® Factor II dPCR has been validated using the reagents included in the kit and those recommended by the dPCR system provider, as indicated in section 6 of this document (*Equipment, reagents and materials not included in the kit*).

It is recommended to use the dPCR reagents provided by the thermal cycler provider. Please contact our Technical Support Team for additional information.

## 10.3 | Product stability

Optimal performance of this product is achieved provided that the recommended storage conditions are applied, as specified in Section 5 (*Content and storage conditions of the kit*) from the reception of the kit until the expiration date associated with each production batch.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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