DNA-TECHNOLOGY

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IVD

For professional use only

Hemochromatosis REAL-TIME PCR Genotyping Kit

INSTRUCTION FOR USE

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1. INTENDED USE

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** is intended for research and diagnostic applications. The **Hemochromatosis REAL-TIME PCR Genotyping Kit** is an *in vitro* Nucleic Acid Test (NAT) – human genotyping-based product. The **Hemochromatosis REAL-TIME PCR Genotyping Kit** is designed to detect and discriminate three genetic polymorphisms associated with hereditary hemochromatosis (OMIM #235200) with an aid of Polymerase Chain Reaction (PCR) method. Samples are human biological materials: peripheral blood.

There are no contradictions for use of the **Hemochromatosis REAL-TIME PCR Genotyping Kit.**

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** can be used in clinical and diagnostic laboratories of medical institutions and research practice.

Potential users: personnel qualified in molecular diagnostics methods and working in the clinical and diagnostic laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

The implemented PCR method is based on amplification of a target DNA sequence.

The detection is based on melting curve analysis.

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** employs fluorescent probes specific to one of two alleles of a gene. The PCR mix contains two distinguishably labelled allele-specific probes bearing reporter fluorescent dyes (Fam and Hex) for each variant of polymorphism. After amplification melting of amplicon-probe complexes is performed. It results in changing fluorescence level and is detected by the real-time thermal cycler and is represented by the software as a graph. If the signal probe is partially complementary to the DNA-target the melting temperature will be less than in case when signal probe is absolute complementary to the DNA-target. The interpretation of results is made based on melting temperatures.

In PCR mix for each polymorphism the system for human genomic DNA amplification is included. It allows to control quantity of human DNA in amplification tube to exclude mistakes in genotyping.

The system for human genomic DNA amplification includes DNA-probe with fluorescent tag (Cy5) and quencher molecule. While being hybridized to a target sequence, fluorescent probes are inactivated (quenched). When the amplicon is synthesized, the probes denature and fluorescent tag is no more quenched and therefore provide fluorescent signal. The intensity of fluorescence is measured by Real-time PCR thermal cycler at every step and analyzed with the software provided. The application of three fluorescent dyes makes it possible to determine two alleles and estimate the amount of genomic DNA simultaneously in one tube. Table 1 shows the detection channels of PCR mix.

Table 1. Detection channels of amplification products

PCR mix	Fam	Hex	Rox	Cy5	Cy5.5
HFE: 187 C>G (H63D)	C	G	-	IC	-
HFE: 193 A>T (S65C)	A	T	-	IC	-
HFE: 845 G>A (C282Y)	G	A	-	IC	-

The automatic analysis is available on “DNA-Technology” made instruments: DTlite or DTprime REAL-TIME Thermal Cyclers for **Hemochromatosis REAL-TIME PCR Genotyping Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is available for download at https://www.dna-technology.com/software.

3. CONTENT

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** contains PCR mix, PCR buffer, Taq-AT-polymerase and mineral oil. The detailed description of content is represented in Table 2.

Table 2. The **Hemochromatosis REAL-TIME PCR Genotyping Kit** content, for R1-H939-N3/4EU

Reagent	Description	Amount	Volume per tube
PCR mix			
1. HFE: 187 C>G (H63D)	Colorless transparent liquid	1 tube	960 µL
2. HFE: 193 A>T (S65C)		1 tube	960 µL
3. HFE: 845 G>A (C282Y)		1 tube	960 µL
PCR buffer	Colorless transparent liquid	1 tube	1.44 mL
Taq-AT-polymerase	Colorless transparent liquid	1 tube	72 µL
Mineral oil	Colorless transparent viscous oily liquid	1 vial	2.88 mL

All components are ready to use and do not require additional preparation for operation.

The kit is intended for single use and designed for 48 tests for **Hemochromatosis REAL-TIME PCR Genotyping Kit.**

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

Blood sampling equipment is required:

- For blood collection: 2.0 or 4.0 mL Vacuette blood collection tubes with anticoagulant, for example, salt of ethylenediaminetetraacetate (EDTA) at a final concentration of 2.0 mg/mL or sodium citrate anticoagulant.

Please use only salt of EDTA or sodium citrate as an anticoagulant, since other substances can provide PCR inhibition.

4.2. DNA extraction and PCR

Preamplification-specimen and control preparation area:

- Biological safety cabinet class II;
- Vortex mixer;
- Refrigerator;
- Nucleic acid extraction kit ("DNA-Technology" made **PREP-GS Genetics** REF P-023/4EU or **PREP-RAPID Genetics** REF P-021/4EU are recommended);
- High speed centrifuge (RCF(g) no less than 16000);
- Thermostat (temperature range 50-98 °C);
- Tube rack for 1.5 mL tubes;

- 1.5 mL tubes;
- Physiological saline solution 0.9% NaCl (Sterile);
- Single channel pipettes (dispensers covering 20-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 200 µL, 1000 µL);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Vortex mixer;
- Refrigerator;
- PCR tube rack for 0.2 mL tubes;
- 0.2 mL tubes;
- Single channel pipettes (dispensers covering 20-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 50 µL, 200 µL, 1000 µL);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Post-Amplification – Amplification detection area:

- Real-time PCR thermal cycler.

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of production.

All components of the **Hemochromatosis REAL-TIME PCR Genotyping Kit**, except the Taq-AT-polymerase, must be stored at temperatures from 2 °C to 8 °C over the storage period. PCR mix must be stored at temperatures from 2 °C to 8 °C and out of light during the storage period. The Taq-AT-polymerase must be stored at temperatures from minus 18 °C to minus 22 °C during the storage period. The excessive temperature and light can be detrimental to product performance.

Transportation is allowed in thermal containers with icepacks by all types of covered transport at temperatures from 2 °C to 8 °C inside the container. It is allowed to transport Taq-AT-polymerase in thermal containers with icepacks at temperatures from 2 °C to 8 °C inside the container, but for no longer than 5 days.

Shelf-life of the kit following the first opening of the primary container:

- components of the kit should be stored at temperatures from 2 °C to 8 °C during the storage period;
- PCR mix for amplification should be stored at temperatures from 2 °C to 8 °C and out of light during the storage period

- Taq-AT-polymerase should be stored at temperatures from minus 18 °C to minus 22 °C during the storage period.

The kit stored in under undue regime should not be used.

An expired the **Hemochromatosis REAL-TIME PCR Genotyping Kit** should not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

The conformity of the **Hemochromatosis REAL-TIME PCR Genotyping Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

Contact our official representative in EU for quality issues of the **Hemochromatosis REAL-TIME PCR Genotyping Kit**.

6. WARNINGS AND PRECAUTIONS

Only personnel trained in the methods of molecular diagnostics and the rules of work in the clinical and diagnostic laboratory are allowed to work with the kit.

Handle and dispose all biological samples, reagents and materials used to carry out the assay as if they were able to transmit infective agents. The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacemet type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. Pipettes used to handle reagents must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. Avoid direct contact with the biological samples reagents and materials used to carry out the assay. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, reverse transcription, PCR-amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.

All the liquid solutions are designed for single use and can not be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits. All laboratory equipment, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should remain in their designated areas. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions. Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products. Never transfer lab coats, gloves and tools from the area designed for amplification/detection of the amplification products to the area designed for

extraction/preparation of amplification reactions. Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be exclusively employed for this specific purpose. Remove PCR waste only in a closed form. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

Do not open the tubes after amplification. Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the PCR mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

Do not use the kit:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- After the expiry date provided.

Significant health effects are **NOT** anticipated from routine use of this kit when adhering to the instructions listed in the current manual.

7. SAMPLES

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** is designed to detect DNA extracted from the peripheral blood.

Sampling, sample processing procedures and storage are carried out in accordance with the instructions to the DNA extraction kit from biological material.

Peripheral blood sampling is carried out in vacuum plastic tube. It may be 2.0 or 4.0 mL Vacuette blood collection tubes with anticoagulant, for example salt of ethylenediaminetetraacetate (EDTA) at a final concentration of 2.0 mg/mL or sodium citrate anticoagulant. After taking the material, it is necessary to mix the blood with anticoagulant turning the tube 2 – 3 times.



It is not allowed to use heparin as an anticoagulant.

Transportation and storage of the samples

Samples may be stored at temperatures from 2 °C to 8 °C for no more than 24 h. When it is impossible to deliver the material in the laboratory during the day, a single freeze-thaw cycle is permitted. The frozen material is allowed to be stored at a temperature of minus 20 °C for one month.



The detailed description of sampling and sample processing procedures as well as sample storage and transportation requirements cited in **PREP-GS Genetics** and **PREP-RAPID Genetics** extraction

kits user manuals.

8. PROCEDURE

DNA extracting from biological material

DNA extraction is carried out according to the extraction kit instructions. **PREP-GS Genetics** and **PREP-RAPID Genetics** extraction kits are recommended. The **PREP-GS Genetics DNA Extraction Kit** is intended for long-term storage of the extracted DNA (up to 6 months). The DNA extracted with aid of **PREP-RAPID Genetics DNA Extraction Kit** should be stored no more than one month. It is allowed to use any kits of reagents registered as a medical device and recommended by manufacturers for the extraction of DNA from the corresponding types of biomaterial.



Independently of DNA extraction kit used, a negative control sample should go through all stages of DNA extraction. Physiological saline solution can be used as a negative control in volumes as indicated.



The quantity of DNA must be at least 1.0 ng per reaction (the Cp parameter for IC must not be more than 32). The violation of this requirement will affect the validity of analysis and void the manufacturer guarantee.

Assay procedure



The reagents and tubes should be kept away from direct sun light.

8.1 Mark the required number of 0.2 mL PCR-tubes for each polymorphism to be tested (one tube for each sample to be tested and one extra for negative control “C-”).

Example: to test 5 samples, mark 15 tubes for samples and 3 tubes for “C-”. The resulting number of tubes is 18.

8.2 Vortex the tubes with PCR mixes for 3-5 seconds, then spin for 1-3 seconds to collect the drops.

8.3 Add 20 µL of corresponding PCR mix into the marked tubes (use a new pipette tip for each type of PCR mix).

8.4 Vortex the tubes with PCR buffer and Taq-AT-polymerase for 3-5 seconds, then spin for 1-3 seconds to collect the drops.



Taq-AT-polymerase must be stored at temperatures from minus 18 °C to minus 22 °C. Room temperature exposure is permitted only for a short time. Remove from freezer just prior to use and place on ice.

8.5 Prepare the mixture of PCR buffer and Taq-AT-polymerase. Add into one tube:

- 10×(N+1) µL of PCR buffer,
- 0.5×(N+1) µL of Taq-AT-polymerase,

N — number of the marked tubes including “C-”.

Example: for simultaneous testing of 5 samples and 1 “C-” in one PCR run, mix 190 µL of PCR buffer and 9.5 µL of Taq-AT-polymerase (calculate final volume for 19 (18+1) tubes).

8.6 Vortex the tube for 3-5 seconds, then spin for 1-3 seconds to collect the drops.



The mixture of PCR buffer and Taq-AT-polymerase must be prepared just prior to use.

8.7 Add 10 µL of PCR buffer and Taq-AT-polymerase mixture into each PCR-tube.

8.8 Add one drop (~20 µL) of mineral oil into each tube. Close the tubes.



Follow the steps listed in pp. 8. 9 – 8.14 within two hours after addition of PCR buffer and Taq-AT-polymerase mix to PCR mix.

8.9 Vortex the tubes with samples and "C-" for 3-5 seconds and spin down drops for 1-3 seconds.

In case of using **PREP-GS Genetics DNA Extraction Kit**. After vortexing centrifuge the tubes with the DNA preparation at RCF(g) 16000 for one minute to precipitate the sorbent. If, after isolation, the supernatant containing the isolated DNA was transferred to new tubes, centrifugation is carried out for 1-3 seconds in a vortex mixer.

Open the tube, add DNA sample (or control sample), then close the tube before proceeding to the next DNA sample to prevent contamination. Close the tubes tightly. Use filter tips.

8.10 Add 5.0 µL of DNA sample into corresponding tubes. Do not add DNA into the "C-" tubes.

8.11 Add 5.0 µL of negative control (C-) which passed whole DNA extraction procedure into "C-" tube into corresponding tube.

8.12 Spin tubes for 3-5 seconds.

8.13 Set the tubes into the Real-time Thermal Cycler.

8.14 Launch the operating software for DT instrument[1]. Add corresponding test[2], specify the number and IDs of the samples and negative control samples. Specify the position of the tubes in the thermal unit (8.13) and run PCR. See Table 3.

The type of the negative control tubes must be specified as "Sample".

Table 3. The PCR program for DTlite and DTprime Thermal Cyclers

Step	Temperature, °C	Min.	Sec.	Number of cycles	Optical measurement	Type of the step
1	80	2	00	1		Cycle
	94	5	00			
2	94	0	30	5		Cycle
	67	0	15		v	
3	94	0	5	45		Cycle
	67	0	15		v	
4	25	0	30	1		Cycle
5	25	0	15	50	v	Melting, Δt=1°C; T_{fin}=75°C
6	10[1]	…	…	Holding		Holding
[1] – holding at 25°C is allowed						

[1] Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.
[2] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

9. CONTROLS

The **Hemochromatosis REAL-TIME PCR Genotyping Kit** contains amplification system for human genomic DNA intended to sample intake control (IC). IC allows to determine sufficiency of the extracted DNA for analysis.

To reveal possible contamination a negative control is required.

A negative control sample should go through all stages of DNA extraction. Physiological saline solution can be used as a negative control sample in volumes indicated in supplied instructions.

The test result is considered valid when genotype is defined.

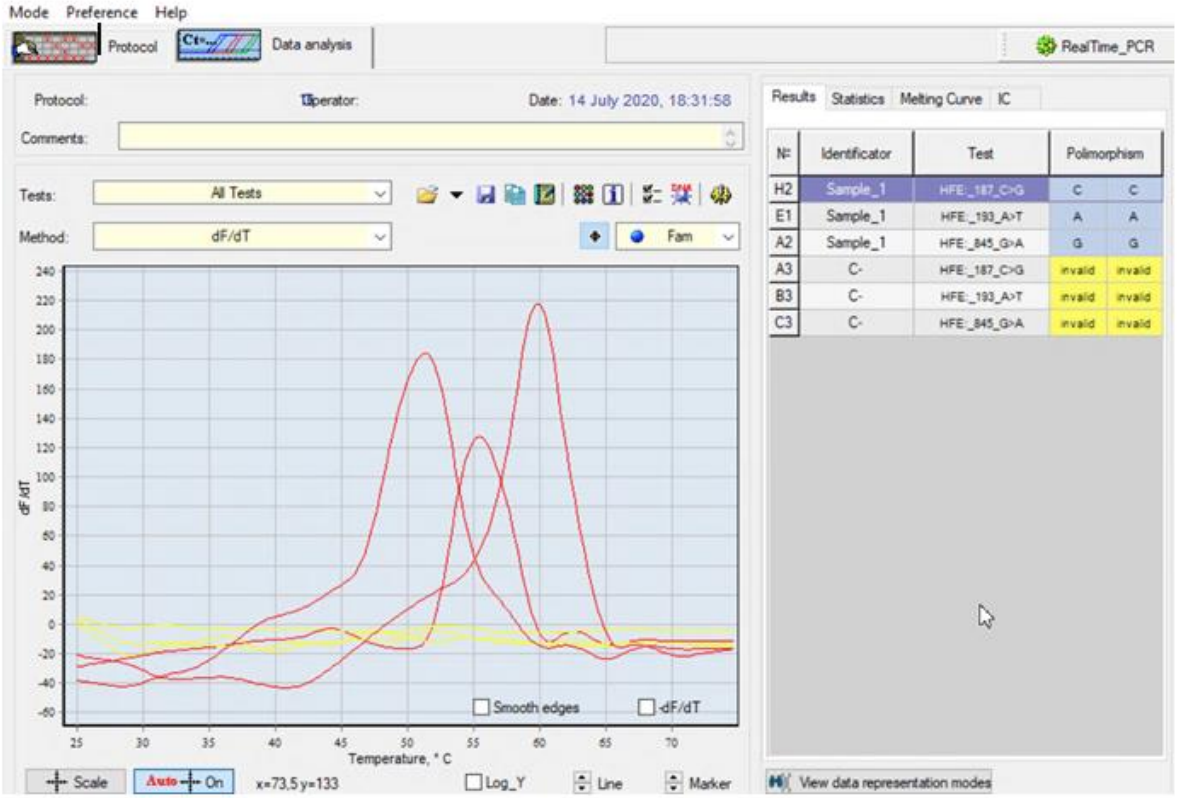
The test result is considered invalid when the Cp of IC (Cy5) is more than 32 or absent.

For negative control samples the software registers invalid result.

If negative control (C-) expresses increasing fluorescence, all tests of the current PCR run are considered false. Decontamination is required.

10. DATA ANALYSIS

Registration and interpretation of the PCR results held in automatic mode. The graph will show the fluorescence dependence of the melting temperature for each tube in the thermoblock. The table will show the sample ID, the name of the polymorphism being detected, and the genotyping result of each sample. It is possible to create and print a report based on the analysis results. Please refer to DTlite or DTprime thermal cycler's user manual for details on working with software.



The software registers the result of human genomic DNA amplification (IC) for all samples. For samples containing a sufficient quantity of DNA for correct analysis, the software defines the genotype, which is displayed in the table in the "Polymorphism" column.
The samples containing an insufficient quantity of DNA (less than 1.0 ng per reaction or Cp>32) will be analyzed as "invalid".

Genotyping report

Logotype

Date 14 July 2020, 18:31:58
Number of tube ...
Patient name ...
Sex ...
Age ...
Organization ...
Clinician name ...
CommentsUnique ...

Information about laboratory

Sample ID: Sample_1

№	Name of research	Results
		Genotype
1	HFE:_187_C>G	C C
2	HFE:_193_A>T	A A
3	HFE:_845_G>A	G G

Study was carried out

Date
Signature

In the case of “invalid” result, PCR method with the same DNA sample, or DNA extraction and PCR, or blood taking (carry out sequentially) is required to repeat.

For samples with sufficient amount of DNA (Cp≤32) the software can register uncertain result according to the following criteria of polymorphism analysis:

?	?

- “Analysis on temperature peak” – – the melting temperature of PCR products differs by more than 3.0 °C from the predetermined one;
- “Differential analysis” - the difference between predetermined and obtained temperatures on Fam and Hex channels for one genotype is more than 2.0 °C;
- “Lower line of temperature peak” - the temperature peak (dF/dT) < 5.0

For such samples retesting is required.

Table 4. Genotypes and melting temperatures (only for DTlite, DTprime instruments)

Polymorphism	Homozygote Fam/Fam			Homozygote Hex/Hex			Heterozygote		
	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C
HFE: 187 C>G (H63D)	CC	49.2	38.4	GG	34.1	49.3	CG	48.8	48.8
HFE: 193 A>T (S65C)	AA	61.5	52.4	TT	54.0	62.1	AT	60.3	60.5
HFE: 845 G>A (C282Y)	GG	57.3	49.3	AA	50.3	56.1	GA	57.2	55.3

DNA-Technology Genotyping assays provide genetic information for some, but not all polymorphic loci known to be associated with certain medical conditions. This information estimates a probability of disease development but does not provide a definitive diagnosis, since other genes may contribute to the odds of disease onset. Moreover, the professional medical consultation regarding complex diseases cannot solely rely on genetic testing. The medical recommendations should also consider behavioral, physical, nutritional and familial information of a patient. On the basis of DNA-Technology Genotyping assays, a specialist can conclude whether a person of a certain genotype has lower or higher chance of disease development in relation to average risk. The definitive diagnosis is a derivative of a physician's experience and the depth of clinical information.

At the assay development stage, we review the most up-to-date scientific literature on genetic associations repeatedly confirmed by independent research. We restrict our genotyping assays to a relatively small set of genetic markers because we believe they provide the most helpful and unbiased information about possible genetic susceptibility to common diseases.

11. SPECIFICATIONS

a. The analytical specificity of the **Hemochromatosis REAL-TIME PCR Genotyping Kit** was assessed by bioinformatics analysis using available on-line databases with up-to-date comprehensive genetic information. The specific oligonucleotides used in the test were checked against GenBank database sequences. None of the sequences showed sufficient similarity for unspecific detection.

b. The lower limit of detection is not less than 1.0 ng of human DNA per amplification tube, which corresponds to Cp≤32 on the IC detection channel. When the amount of DNA is smaller (Cp>32 on the IC detection channel), the manufacturer does not guarantee the correct result of the kit.

After the amplification reaction for samples with insufficient quantity of DNA (less than 1.0 ng per amplification tube), the result is defined as unreliable.

The claimed specifications are guaranteed when DNA extraction is performed with **PREP-GS Genetics** REF P-023/4EU or **PREP-RAPID Genetics** REF P-021/4EU extraction kits.

12. TROUBLESHOOTING

Table 5. Troubleshooting

	Result	Possible cause	Solution
C-	+	Contamination	Dispose current batch Perform decontamination procedures
IC	Invalid	PCR inhibition Insufficient amount of DNA	Repeat whole test Resample

If you face to any issues not described, contact our customer service department:

Phone: +7(495) 640.16.93

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com/support

13. QUALITY CONTROL

The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- quality management in manufacturing processes;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Contact our customer service by quality issues of **Hemochromatosis REAL-TIME PCR Genotyping Kit**.

Technical support:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

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Phone/fax: +7(495) 640.17.71

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https://www.dna-technology.com

14. KEY TO SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version		Do not reuse
	Non-sterile		Caution

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