DNA-TECHNOLOGY

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IVD

For professional use only

Hemostasis REAL-TIME PCR Genotyping Kit

INSTRUCTION FOR USE

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1. INTENDED USE

Hemostasis REAL-TIME PCR Genotyping Kit is intended for research and diagnostic applications.

The **Hemostasis REAL-TIME PCR Genotyping Kit** is an in vitro Nucleic Acid Test (NAT) – human genotyping-based product.

Hemostasis REAL-TIME PCR Genotyping Kit is designed to detect polymorphisms of genes associated with the risk of hemostasis disorders (bleeding, thrombosis): F2 (prothrombin, coagulation factor II, 20210 G>A); F5 (coagulation factor V Leiden, 1691 G>A); F7 (coagulation factor VII, 10976 G>A); F13 (coagulation factor XIII G>T); FGB (fibrinogen beta chain, coagulation factor I, -455 G>A); ITGA2 (glycoprotein Ia, VLA-2 receptor 807 C>T); ITGB3 (integrin beta 3, platelet fibrinogen receptor 1565 T>C); SERPINE1 (plasminogen activator inhibitor type I, - 675 5G>4G) by real-time PCR in human biological material (whole peripheral blood, dry blood spots (DBS), buccal epithelium).

Functional purpose: *in vitro* diagnostics, screening.

Indications for use: diagnosis of genetic predisposition to dyscrasia; screening of pregnant women.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **Hemostasis REAL-TIME PCR Genotyping Kit**.

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

Potential users: qualified personnel trained in molecular diagnostic methods.

Apply the kit only as directed in the present instruction for use.

2. METHOD

Polymerase chain reaction (PCR) with real-time results detection, melting curves analysis, qualitative analysis.

The kit functions on the basis of PCR DNA amplification principle. Amplification process consists of a series of repeated DNA temperature denaturation cycles, annealing of primers complementary to a specific DNA site, and the sequential elongation of polynucleotide chains from these primers by Taq polymerase.

Signal probes containing Fam and Hex fluorescent tags for each variant of genetic polymorphism to be detected are introduced into PCR mix. After PCR is completed, a round of temperature melting of duplexes formed by amplicons and signal probes is performed, resulting in a change of fluorescence level, which is recorded and presented as a graph by the instrument software. If signal probe is partially complementary to DNA target, melting temperature of such duplex will be lower than in the case of full complementarity of the probe. Analysis results are interpreted based on signal probes melting temperature.

Composition of PCR mixes specific for each genetic polymorphism includes internal control (IC) — system for human genomic DNA amplification which allows to control the amount of DNA in amplification tube and to exclude genotyping failures.

System of human genomic DNA amplification includes DNA probe containing fluorescent tag (Cy5) and fluorescence quencher. When specific product is formed, DNA probe is destroyed and the effect of the quencher on the fluorescent tag stops, which results in a rise in fluorescence level. The number of destroyed probes (and therefore fluorescence level) increases in proportion to the number of specific amplicons formed and changes during each amplification cycle.

Hemostasis REAL-TIME PCR Genotyping Kit contains PCR mix specific for each genetic polymorphism. The list of gene polymorphisms and possible genotypes detected by the kit is presented in Table 1.

Table 1. List of possible genotypes determined by the **Hemostasis REAL-TIME PCR Genotyping Kit**

Polymorphism	Genotype		
	Frequent homozygous	Rare homozygous	Heterozygote
F2: 20210 G>A	GG	AA	GA
F5: 1691 G>A (Arg506Gln)	GG	AA	GA
F7: 10976 G>A (Arg353Gln)	GG	AA	GA
F13: G>T (Val34Leu)	GG	TT	GT
FGB: -455 G>A	GG	AA	GA
ITGA2: 807 C>T (Phe224 Phe)	CC	TT	CT
ITGB3: 1565 T>C (Leu33Pro)	TT	CC	TC
SERPINE1 (PAI-1): -675 5G>4G	5G5G	4G4G	5G4G

Using three fluorescent dyes allows to simultaneously determine two alleles and estimate the amount of genomic DNA in one tube.

Using several fluorescent dyes allows to reduce the number of tubes as it provides the possibility to simultaneously register the results of different amplification reactions occurring in one tube. Table 2 shows the detection channels of amplification products.

Table 2. Detection channels of amplification products

Polymorphism (PCR mix)	Detection channels for allele variants and internal control*				
	Fam	Hex	Rox	Cy5	Cy5.5
F2: 20210 G>A	G	A	-	IC	-
F5: 1691 G>A (Arg506Gln)	G	A	-	IC	-
F7: 10976 G>A (Arg353Gln)	G	A	-	IC	-
F13: G>T (Val34Leu)	G	T	-	IC	-
FGB: -455 G>A	G	A	-	IC	-
ITGA2: 807 C>T (Phe224Phe)	C	T	-	IC	-
ITGB3: 1565 T>C (Leu33Pro)	T	C	-	IC	-
SERPINE1 (PAI-1): -675 5G>4G	5G	4G	-	IC	-
* – the internal control (IC) system, which is a human genomic DNA amplification system, allows for the assessment of DNA presence in the amplification tube and helps prevent genotyping errors.					

To control the detection of frequent and rare homozygous, control samples for the **Hemostasis REAL-TIME PCR Genotyping Kit** are used.

Control samples are mixes of cloned gene regions detected using this reagent kit in frequent or rare homozygous states which are meant for assay quality control (detection of frequent and rare homozygotes) by the end user of the kit.

WARNING! Control samples are compatible with **Hemostasis REAL-TIME PCR Genotyping Kit** only.

Table 3. **Hemostasis REAL-TIME PCR Genotyping Kit** control samples

Control sample	Controlled PCR mix	Expected genotyping result
Frequent homozygous	F2: 20210 G>A	GG
	F5: 1691 G>A (Arg506Gln)	GG
	F7: 10976 G>A (Arg353Gln)	GG
	F13: G>T (Val34Leu)	GG
	FGB: -455 G>A	GG
	ITGA2: 807 C>T (Phe224 Phe)	CC
	ITGB3: 1565 T>C (Leu33Pro)	TT
	SERPINE1 (PAI-1): -675 5G>4G	5G5G
Rare homozygous	F2: 20210 G>A	AA
	F5: 1691 G>A (Arg506Gln)	AA
	F7: 10976 G>A (Arg353Gln)	AA
	F13: G>T (Val34Leu)	TT
	FGB: -455 G>A	AA
	ITGA2: 807 C>T (Phe224 Phe)	TT
	ITGB3: 1565 T>C (Leu33Pro)	CC
	SERPINE1 (PAI-1): -675 5G>4G	4G4G

Automatic analysis for **Hemostasis REAL-TIME PCR Genotyping Kit** is available on instruments manufactured by "DNA-Technology": DTprime, DTprime II, DTlite with 4 detection channels or more. (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of software is available for download at https://www.dna-technology.com/software.

3. CONTENTS

The **Hemostasis REAL-TIME PCR Genotyping Kit** content is represented in Table 5.

Table 4. The **Hemostasis REAL-TIME PCR Genotyping Kit** content, package N R1-H957-N3/4EU

Reagent	Description	Amount	Volume per tube/vial
PCR mix (8 items):	Colorless or pink transparent liquid		
• F2: 20210 G>A		1 tube	960 µL
• F5: 1691 G>A (Arg506Gln)		1 tube	960 µL
• F7: 10976 G>A (Arg353Gln)		1 tube	960 µL
• F13: G>T (Val34Leu)		1 tube	960 µL
• FGB: -455 G>A		1 tube	960 µL
• ITGA2: 807 C>T (Phe224Phe)		1 tube	960 µL
• ITGB3: 1565 T>C (Leu33Pro)		1 tube	960 µL
• SERPINE1 (PAI-1): -675 5G>4G		1 tube	960 µL
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	1 tube	200 µL
PCR buffer	Colorless transparent liquid	4 tubes	1.0 mL in each
Mineral oil	Colorless transparent viscous oily liquid	1 vial	8.0 mL
Control sample (frequent homozygous)	Colorless transparent liquid	1 tube	480 µL
Control sample (rare homozygous)	Colorless transparent liquid	1 tube	480 µL

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

Hemostasis REAL-TIME PCR Genotyping Kit in package N is designed for 48 tests (no more than 12 runs), including analysis of test samples, negative controls and control samples.

4. ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED

The following equipment, reagents and consumables are required:

- UV PCR cabinet;
- real-time detecting thermal cycler (hereinafter – detecting thermal cycler; the required parameters are indicated below);
- vortex mixer (DTspin laboratory shaker (DNA-Technology, Russia) is recommended);
- refrigerator with freezer;
- 1.5 mL tubes with caps;
- 0.2 mL PCR tubes with caps or 0.2 mL strips of 8 with caps;
- tube rack for 1.5 mL tubes;
- tube rack for 0.2 mL tubes or strips;
- single channel pipettes (dispensers covering 0.5–10 µL, 2.0–20 µL, 10–100 µL, 20–200 µL, 100–1,000 µL, 200–1,000 µL volume range);
- RNase and DNase free aerosol barrier pipette tips (volume 10 µL, 20 µL, 200 µL, 1,000 µL);
- powder-free surgical gloves;
- container for used pipette tips, tubes and other consumables;
- physiological saline solution 0.9% NaCl (sterile) (if necessary);
- nucleic acid extraction kit (DNA-Technology, Russia): **PREP-GS Genetics**, **PREP-RAPID Genetics**, **PREP-OPTIMA**, **PREP-OPTIMA MAX, PREP-MB MAX, PREP-CITO DBS**; **PREP-MB-DBS DWP**
- **Sample Intake Control REAL-TIME PCR Kit** (if necessary).

The following detecting thermal cyclers are validated for work with the **Hemostasis REAL-TIME PCR Genotyping Kit**:

- DTprime in DTprime *M* modification (manufactured by "DNA-Technology R&P", LLC), hereinafter – DTprime;
- DTprime II in DTprime II *M* modification (manufactured by "DNA-Technology R&P", LLC), hereinafter – DTprime II;
- DTlite in DTlite *S* modification (manufactured by "DNA-Technology R&P", LLC), hereinafter – DTlite.

For the use of detecting thermal cyclers other than those listed above, please consult the reagent kit manufacturer for consultation.

Software:

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

5. TRANSPORT AND STORAGE CONDITIONS

Expiry date – 12 months from the date of manufacture.

5.1. Storage conditions

- All components of **Hemostasis REAL-TIME PCR Genotyping Kit**, except for TechnoTaq MAX polymerase, must be stored in a refrigerator or a cooling chamber at temperatures from 2°C to 8°C over the storage period.
- PCR mix must be stored away from light over the storage period.
- TechnoTaq MAX polymerase must be stored in a freezer at temperatures ranging from minus 22°C to minus 18°C over the storage period.

WARNING! The excessive temperature and light can be detrimental to product performance.

5.2. Transport conditions

Transportation of the reagent kit is carried out in thermoboxes with ice packs by all types of roofed transport at the temperature inside the container corresponding to the storage conditions of the kit components.

- It is allowed to transport the kit, except for TechnoTaq MAX polymerase, in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes from 2°C to 25°C for no longer than 5 days.
- It is allowed to transport TechnoTaq MAX polymerase in thermoboxes with ice packs by all types of roofed transport at the temperature inside the thermoboxes up to 25°C for no longer than 5 days.

WARNING! Reagent kits transported with violation of temperature conditions must not be used.

5.3. Shelf-life of the kit following the first opening of the primary container

- All components of the kit, except for TechnoTaq MAX polymerase, must be stored in a refrigerator or a cooling chamber at temperatures from 2°C to 8°C over the storage period.
- PCR mix must be stored in a refrigerator or a cooling chamber at temperatures from 2°C to 8°C and out of light over the storage period.
- TechnoTaq MAX polymerase must be stored at temperatures from minus 22°C to minus 18°C over the storage period.

WARNING! Kits stored with violation of storage conditions must not be used.

An expired **Hemostasis REAL-TIME PCR Genotyping Kit** must not be used.

We strongly recommend following the current instructions for use in order to obtain accurate and reliable results.

The manufacturer guarantees the conformity of **Hemostasis REAL-TIME PCR Genotyping Kit** to the technical documentation if the storage, transportation and handling requirements are fulfilled.

6. WARNINGS AND PRECAUTIONS

- Molecular biology procedures, such as nucleic acid extraction, PCR-amplification and detection require qualified staff to avoid the risk of erroneous or unreliable results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.
- Wear powder-free single-use surgical gloves. Wear work clothes and personal protective equipment while working with pathogenic microorganisms. The work clothes and personal protective equipment must be suitable for work to be performed and comply with health and safety requirements.
- Avoid any direct contact with the biological samples, reagents and materials used to carry out the test. Avoid producing spills or generating aerosols. Do not eat/drink components of the kit. Do not inhale gas/fumes/vapor/aerosols produced by the components of the kit. Avoid contact with eyes.
- Samples must be handled under a laminar flow hood.
- Pipettes used to handle samples must only be used for one purpose. The pipettes must be of positive displacement type or be used with aerosol barrier pipette tips.

- The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way to be utilized in a single session.
- Handle and dispose of all biological samples, reagents and materials used to carry out the assay as if infectious[1, 2]. Any material being exposed to biological samples must be treated with disinfecting solution for at least 30 min or autoclaved for 1 hour at 121°C before disposal.
- All of the liquid solutions are designed for single use and cannot be used more than once in amplification reactions.
- Only use the reagents provided in the kit and those recommended by the manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits.
- All laboratory equipment and tools, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, gloves, etc., as well as reagents must be strictly stationary. 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 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. Never introduce amplification products in the area designed for extraction/preparation of amplification reactions.
- Do not open the tubes after amplification. Avoid producing accidental spills of the amplification products. Dispose of all PCR waste materials (tubes, tips etc.) only in a closed form in a specialized sealed container with disinfectant solution. Waste materials must be removed in accordance with laboratory internal procedures, and with national and international standards.
- Working surfaces, as well as rooms where NA extraction and PCR are performed, must be disinfected with bactericidal irradiators (UVGI) for 30 min before and after the assay. All surfaces in the laboratory (test tube racks, equipment, tools, etc.) must be treated with disinfecting solution daily.

Emergency actions

Eye Contact: If any component of the kit enters the eyes, flush the eyes gently using potable running water for 15 min or longer, making sure that the eyelids are held open. If pain or irritation occurs, seek medical attention.

Skin Contact: If any component of this kit comes into contact with the skin and causes discomfort, remove any contaminated clothing. Rinse the affected area with plenty of soap and water. If pain or irritation occurs, seek medical attention.

Ingestion: If any component of this kit is ingested, rinse the mouth with plenty of potable water. If irritation or discomfort occurs, seek medical attention.

Do not use the kit:

- if the transportation and storage conditions have been violated;
- if the appearance of the reagents does not correspond to the product documentation;
- if the packaging of the kit components is breached;
- after the expiry date of the kit.

Adverse health effects are **NOT** anticipated from routine use of this kit in compliance with the current instruction for use.

7. SAMPLES

The **Hemostasis REAL-TIME PCR Genotyping Kit** is designed to detect DNA extracted from whole peripheral blood, dried blood spots (DBS) and buccal epithelium.

Method limitations: intravenous injections of heparin, infusions of parenteral nutrition less than 6 hours

[1] - All oligonucleotide components are produced by artificial synthesis in compliance with internal quality control protocol. They do not contain blood or products of blood processing.

[2] - Positive control is produced using artificial DNA synthesis technology, it does not contain parts of infectious agents.

before the test.

Sample collection, pretreatment and storage are performed according to the DNA extraction reagent kits for the corresponding biomaterial.

Table 5. Compatibility of DNA extraction kits with biomaterial

DNA extraction kit	Biomaterial		
	Whole peripheral blood	**DBS**	**Buccal epithelium**
PREP-GS Genetics	yes	no	no
PREP-RAPID Genetics	yes	no	no
PREP-MB MAX	yes	no	no
PREP-CITO DBS	no	yes	no
PREP-OPTIMA	no	no	yes
PREP-OPTIMA MAX	yes	no	no
PREP-MB-DBS DWP	no	yes	no

7.1. General requirements

The quality of sample collection, sample storage, transport and pretreatment is crucial for obtaining correct results. Incorrect sample collection may lead to unreliable results and, therefore, to the need to repeat the sample collection procedure.

Use sterile RNase- and DNase-free aerosol barrier pipette tips during biomaterial preparation and NA extraction.

To prevent contamination, only open one tube cap at a time and close it before proceeding to the next tube. Tubes containing different samples must never be opened at the same time.

7.2. Sample collection

7.2.1. Whole peripheral blood collection

Whole peripheral blood is collected into Vacuette type 2.0 mL or 4.0 mL vacuum plastic tubes with ethylenediaminetetraacetate (EDTA) salt as an anticoagulant in the final concentration of 2.0 mg/mL. Sodium citrate may also be used as an anticoagulant. To mix blood with anticoagulant, invert the tube 2–3 times after biomaterial collection.

Method limitations: Intravenous injections of heparin, infusions of parenteral nutrition products less than 6 hours before the test.

7.2.2. Obtaining dried blood spots (DBS)

The blood is applied to the sampling paper in an amount sufficient to obtain DBS with a diameter of at least 1.0 cm, and the blood must soak through the paper. After applying the sample, the filter card is dried horizontally on a clean, degreased surface for at least two hours without any additional heat treatment and exposure to direct sunlight.

WARNING! To avoid incorrect results, no one other than the patient should handle the sampling paper without wearing gloves.

Using a special puncher, punch out 3 discs, 3.0 mm in diameter, from the central part of the DBS and place them in a 1.5 mL tube.

WARNING! It is recommended to punch the discs immediately before the DNA extraction procedure.

7.2.3. Buccal epithelium collection

WARNING! Sampling procedure is carried out using a dry swab. It is necessary to keep the solutions away

from skin, eyes, and mucous membranes. If the contact occurs, it is necessary to wash the affected area immediately and obtain medical attention.

Sampling procedure is carried out using special sterile disposable instruments – swabs, cytobrushes or tampons in accordance with established procedure.

It is recommended to abstain from eating, smoking, and taking medication perorally two hours prior the biological material sampling. Rinse mouth with water twice before sampling.

Order of taking:

1. Open the package containing the sterile disposable swab, holding it by the handle and avoiding contact with the tip.
2. Place the swab in the oral cavity and thoroughly rub the inner surface of one cheek, then the other, with gentle pressure for 30 sec (25–30 times) while rotating the swab.
3. After collecting the biological material, transfer the swab into a 1.5 mL tube containing transport medium or lysis solution.
4. Withdraw the swab from the solution while pressing it against the side of the tube to expel excess liquid, remove the swab, and discard it.
5. Close the tube tightly and mark it.

7.3. Transport and storage of samples

7.3.1 Dried blood spots (DBS)

DBS can be stored for up to one year after drying in individual packages in low humidity conditions (max. 30%) at 2°C to 8°C. For longer storage periods, samples of DBS should be stored as recommended by the filter card manufacturer.

7.3.2 Whole peripheral blood, buccal epithelium

Whole peripheral blood and buccal epithelium must be transported and stored according to the instructions to the used NA extraction kits. In case there are no recommendations for these types of biomaterial, follow these conditions:

1. Whole peripheral blood can be stored at 2°C to 8°C for no more than 24 hours. If the material cannot be delivered to the laboratory within 24 hours, it can be frozen once. Frozen material can be stored at minus 20°C for up to one month.
2. Buccal epithelium can be stored at 2°C to 8°C for no more than 24 hours. If the material cannot be delivered to the laboratory within 24 hours, it can be frozen once. Frozen material can be stored at minus 20°C for up to one month.

8. PROCEDURE

8.1. DNA extraction from biomaterial

DNA extraction is carried out according to the extraction kit instructions. **PREP-GS Genetics**, **PREP-RAPID Genetics**, **PREP-OPTIMA**, **PREP-OPTIMA MAX**, **PREP-MB MAX**, **PREP-CITO DBS** and **PREP-MB-DBS DWP** extraction kits are recommended.

WARNING! Run a negative control alongside DNA extraction through all preparation stages. Use physiological saline solution (in volumes per the extraction kit instructions) or the kit provided negative control.

General requirements:

- Follow the DNA preparation recommendations from the NA extraction kit's instruction.
- Use filter tips.
- Open tube caps only when introducing the DNA sample, then close immediately.

- If using strips: close the strip cap after introducing samples before proceeding to the next strip.
- Ensure all tubes are tightly closed after dosing.

For DNA preparations after storage, prepare them as follows before adding to the amplification mixture:

- **PREP RAPID Genetics, PREP CITO DBS, PREP OPTIMA, PREP OPTIMA MAX**: vortex (3–5 sec), then centrifuge on vortex (1–3 sec).
- **PREP GS Genetics**: vortex (5–10 sec), incubate at 50 °C (5 min) (if the eluate was refrigerated), then centrifuge at 16 000 RCF(g) (1 min).
- **PREP MB MAX**: centrifuge on vortex carefully (without shaking, 1–3 sec), place into magnetic rack. If supernatant transferred to new tubes — centrifuge (1–3 sec) after shaking.
- **PREP MB DBS DWP**: mix by pipetting (3–5 times) before adding to amplification mixture.

8.2. PCR preparation

WARNING! Reagents and tubes should be kept away from direct sunlight.

8.2.1 Mark the required number of 0.2 mL PCR tubes or stripped tubes for each polymorphism (one tube for each test sample, one for negative control C- and one for each control sample.

Example: there are 5 samples to be tested for 8 polymorphisms. Mark 8 tubes per sample (one for each polymorphism) – 40 for tubes test samples, 8 tubes for negative control, and 16 tubes for two control samples. The total number of tubes for all polymorphisms is 64.

8.2.2 Vortex the tubes with PCR mixes for 3–5 sec, then centrifuge for 1–3 sec on vortex.

8.2.3 Add 20 µL of the corresponding PCR mix into the marked tubes.

8.2.4 Vortex the tubes with PCR buffer and TechnoTaq MAX polymerase for 3–5 sec, then centrifuge for 1–3 sec on vortex.

WARNING! TechnoTaq MAX polymerase must be got out from the freezer immediately prior to use.

8.2.5 Prepare the mixture of PCR buffer and TechnoTaq MAX polymerase. Add into one tube:

- 10 × (N+1) µL of PCR buffer,
- 0.5 × (N+1) µL of TechnoTaq MAX polymerase,

where **N** is the number of the marked tubes including C- and control samples.

Example: There are 5 samples to be tested for 8 polymorphisms. The number of marked tubes is 64. The mixture of PCR buffer and TechnoTaq MAX polymerase should be prepared for 65 (64+1) tubes, i.e., 650 µL of PCR buffer must be mixed with 32.5 µL of TechnoTaq MAX polymerase.

8.2.6 Vortex the tube with mixture of PCR buffer and TechnoTaq MAX polymerase for 3–5 sec, then centrifuge for 1–3 sec on vortex.

WARNING! The mixture of PCR buffer and TechnoTaq MAX polymerase must be prepared immediately prior to use.

8.2.7 Add 10 µL of PCR buffer and TechnoTaq MAX polymerase mixture into each tube with PCR mix.

WARNING! Follow the steps listed in pp. 8.2.8–8.2.15 within two hours after adding PCR buffer and TechnoTaq MAX polymerase mixture to PCR mix.

8.2.8 Add one drop (~20 µL) of mineral oil into each tube. Cap the tubes/strips loosely.

8.2.9 Vortex the tube with control samples for 3–5 sec and centrifuge for 1–3 sec on vortex.

8.2.10 Add 5.0 µL of DNA sample into corresponding tubes. Do not add DNA into the C- and the control sample tubes.

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

8.2.12 Add 5.0 µL of the control samples into each corresponding control sample tube.

8.2.13 Centrifuge the tubes/strips for 1-3 sec on vortex.

8.2.14 Set the tubes/strips into real-time thermal cycler.

8.2.15 Launch the operating software for DT instrument[3]. Add corresponding test[4], specify the number and IDs of the samples, negative control and control samples. Specify the position of the tubes/strips in the thermal unit (8.2.14) and run PCR. See Table 7.

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

Table 6. The PCR program for DTlite, DTprime and DTprime II 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	25**	…	…	Temperature hold		Temperature hold
* – When creating step 5, Check the "Melting curve" type. ** – Temperature hold at 10°C is allowed.						

9. CONTROLS

The **Hemostasis REAL-TIME PCR Genotyping Kit** contains amplification system for human genomic DNA which serves as internal control (IC). IC allows to determine sufficiency of the extracted DNA for analysis. The test result is considered invalid when the Cp of IC (Cy5) is more than 32 or absent.

To reveal possible contamination, a negative control is required.

WARNING! A negative control should go through all stages of DNA extraction. Physiological saline solution or negative control from the extraction kit can be used as a negative control in volumes indicated in supplied instructions.

The test result is considered valid when genotype is defined.

In case of obtaining positive result (genotype determination) for negative control, all results of the current PCR run are considered false. In this case, conduction of special procedures against possible contamination is required.

10. DATA ANALYSIS

[3] - Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.

[4] - Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

10.1 PCR results are registered and interpreted automatically. 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. For detailed instructions on software operation, please consult the user manual for the DTlite or DTprime thermal cycler.

10.2 The software records human genomic DNA amplification (IC) results for all samples. For the correct functioning of the kit, the amount of the tested DNA must be at least 1.0 ng per amplification tube, which corresponds to Cp ≤ 32.0.

10.3 For samples with sufficient amount of DNA, the software defines the genotype (displayed in the "Polymorphism" column).

10.4 The corresponding genotype (see Table 3), which is shown in the table in the column "Polymorphism", must be determined in the control samples.

10.5 The samples containing an insufficient quantity of DNA (less than 1.0 ng per reaction or Cp > 32) will be analyzed as "**invalid**". In this case:

- Repeat PCR with the same DNA sample;
- Repeat DNA extraction and PCR;
- Obtain a second biomaterial sample and repeat the analysis.

10.6 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 from predetermined more than on 3.0°C (see table 8);
- "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 repeating PCR is required.

10.7 For the negative control, an "**invalid**" result is the expected (normal) outcome recorded by the software.

10.8 If a positive result (genotype determination) was obtained for negative control, the results of the whole run batch are considered invalid. In this case, special measures for detection and elimination of possible contamination are required.

Table 7. Genotypes and melting temperatures (only for DTlite, DTprime and DTprime II instruments)

Polymorphism	Homozygote Fam/Fam			Homozygote Hex/Hex			Heterozygote		
	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C	Genotype	Fam, °C	Hex, °C
F2: 20210 G>A	**GG**	59.5	47.0	**AA**	48.0	58.5	**GA**	57.4	57.7
F5: 1691 G>A (Arg506Gln)	**GG**	54.4	48.7	**AA**	39.4	54.4	**GA**	52.6	52.9
F7: 10976 G>A (Arg353Gln)	**GG**	52.9	45.7	**AA**	31.6	52.5	**GA**	51.5	52.1
F13: G>T (Val34Leu)	**GG**	59.5	44.0	**TT**	46.0	57.0	**GT**	58.0	56.0
FGB: -455 G>A	**GG**	50.3	43.9	**AA**	42.9	50.2	**GA**	49.5	49.7
ITGA2: 807 C>T (Phe224Phe)	**CC**	52.0	42.0	**TT**	43.0	52.0	**CT**	51.0	51.0
ITGB3: 1565 T>C (Leu33Pro)	**TT**	58.2	47.2	**CC**	46.8	55.5	**TC**	57.0	55.5
SERPINE1:(PAI-1): -675 5G>4G	**5G5G**	52.1	46.6	**4G4G**	39.6	54.4	**5G4G**	50.4	52.5

11. SPECIFICATIONS

a. Limit of detection

Analytical specificity of the **Hemostasis 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.

The limit of detection is at least 1.0 ng of human DNA per amplification tube, which corresponds to 300 DNA copies (Cp ≤ 32.0 on the IC (Cy5) detection channel). When the amount of DNA is smaller (Cp > 32.0 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 invalid.

b. Diagnostic characteristics

Diagnostic sensitivity and diagnostic specificity values are calculated as a proportion of correctly determined alleles detected among the entire pool of alleles determined by the **Hemostasis REAL-TIME PCR Genotyping Kit**.

Polymorphism F2: 20210 G>A

Biological material	F2: 20210 G>A		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	A	G		
	rare	frequent		
Whole peripheral blood	3	107	100% (29.24-100)	100% (96.61-100)
Buccal epithelium	1	49	100% (2.50-100)	100% (92.75-100)
Dried blood spots	1	49	100% (2.50-100)	100% (92.75-100)
Total	5	205	100% (47.82-100)	100% (98.22-100)

Polymorphism F5: 1691 G>A (Arg506Gln)

Biological material	F5: 1691 G>A (Arg506Gln)		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	A	G		
	rare	frequent		
Whole peripheral blood	3	107	100% (29.24-100)	100% (96.61-100)
Buccal epithelium	1	49	100% (2.50-100)	100% (92.75-100)
Dried blood spots	1	49	100% (2.50-100)	100% (92.75-100)
Total	5	205	100% (47.82-100)	100% (98.22-100)

Polymorphism F7: 10976 G>A (Arg353Gln)

Biological material	F7: 10976 G>A (Arg353Gln)		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	A	G		
	rare	frequent		
Whole peripheral blood	18	92	100% (81.47-100)	100% (96.07-100)
Buccal epithelium	5	45	100% (47.82-100)	100% (92.13-100)
Dried blood spots	7	43	100% (59.04-100)	100% (91.78-100)
Total	30	180	100% (88.43-100)	100% (97.97-100)

Polymorphism F13: G>T (Val34Leu)

Biological material	F13: G>T (Val34Leu)		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	T	G		
	rare	frequent		
Whole peripheral blood	36	74	100% (90.26-100)	100% (95.14-100)
Buccal epithelium	12	38	100% (73.53-100)	100% (90.75-100)
Dried blood spots	9	41	100% (66.37-100)	100% (91.40-100)
Total	57	153	100% (93.73-100)	100% (97.62-100)

Polymorphism FGB: -455 G>A

Biological material	FGB: -455 G>A		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	A	G		
	rare	frequent		
Whole peripheral blood	29	81	100% (88.06-100)	100% (95.55-100)
Buccal epithelium	7	43	100% (59.04-100)	100% (91.78-100)
Dried blood spots	12	38	100% (73.53-100)	100% (90.75-100)
Total	48	162	100% (92.60-100)	100% (97.75-100)

Polymorphism ITGA2: 807 C>T (Phe224 Phe)

Biological material	ITGA2: 807 C>T (Phe224 Phe)		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	T	C		
	rare	frequent		
Whole peripheral blood	41	69	100% (91.40-100)	100% (94.79-100)
Buccal epithelium	23	27	100% (85.18-100)	100% (87.23-100)
Dried blood spots	15	35	100% (78.20-100)	100% (90.00-100)
Total	79	131	100% (95.44-100)	100% (97.22-100)

Polymorphism ITGB3: 1565 T>C (Leu33Pro)

Biological material	ITGB3: 1565 T>C (Leu33Pro)		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	C	T		
	rare	frequent		
Whole peripheral blood	17	93	100% (80.49-100)	100% (96.11-100)
Buccal epithelium	8	42	100% (63.06-100)	100% (91.59-100)
Dried blood spots	6	44	100% (54.07-100)	100% (91.96-100)
Total	31	179	100% (88.78-100)	100% (97.96-100)

Polymorphism SERPINE1 (PAI-1): -675 5G>4G

Biological material	SERPINE1 (PAI-1): -675 5G>4G		Diagnostic sensitivity (%)	Diagnostic specificity (%)
	Allele			
	4G	5G		
	rare	frequent		
Whole peripheral blood	64	46	100% (94.40-100)	100% (92.29-100)
Buccal epithelium	32	18	100% (89.11-100)	100% (81.47-100)
Dried blood spots	29	21	100% (88.06-100)	100% (83.89-100)
Total	125	85	100% (97.09-100)	100% (95.79-100)

Diagnostic sensitivity 100% (93.73-100).

Diagnostic specificity 100% (97.62-100).

c. Within-batch and between-batch precision

Within-batch precision (95% CI) is 100% (83.16–100%).

Between-batch precision (95% CI) is 100% (83.16–100%).

NOTE. The claimed specifications are guaranteed when DNA extraction is performed with **PREP-GS Genetics, PREP-RAPID Genetics, PREP-OPTIMA, PREP-OPTIMA MAX, PREP-MB MAX, PREP-CITO DBS** and **PREP-MB-DBS DWP** extraction kits.

12. TROUBLESHOOTING

Table 8. Troubleshooting

	Result	Possible cause	Solution
Control samples	Genotype is not defined	Operation error PCR inhibition Violation of storage and handling requirements	Repeat whole test Dispose of the current batch
C-	Genotype is defined	Operation error Contamination	Dispose of the current batch Perform decontamination procedures
IC	Invalid	PCR inhibition Insufficient amount of DNA	Repeat whole test Resample

If you face to any undescribed issues contact our customer service department regarding quality issueswith the kit:

E-mail: hotline@dna-technology.ru

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

13. QUALITY CONTROL

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

Technical support:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

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14. KEY SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
Σ	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
NON STERILE	Non-sterile		