Labcare de Colombia

SGS (CO16/6735)

IV. HUMAN GENETICS

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One of the most advanced areas of PCR diagnostics is the study of the human genome. Particularly topical is research data, which is obtained by using molecular genetic techniques, for preventing and controlling non-communicable diseases (NCD), especially cancer, cardiovascular diseases, respiratory diseases and diabetes. They account for 80 % of all deaths in the NCD group and for over 60 % of deaths (36 million per year) throughout the world.

The World Health Organization predicts that 52 million people will die from NCDs by 2030, with economic losses amounting to 47 trillion dollars on average.

In this regard, in 2011 the UN General Assembly adopted the Political Declaration on Prevention and Control of Non communicable Diseases – the single global long-term strategy to fight NCDs, which was supported by 150 states, including Russia.

12. TYPES OF PCR DIAGNOSIS OF HUMAN GENOME

DNA-Technology offers equipment and kits in the following areas for human genotyping:

- **Study of human major histocompatibility complex (HLA typing)**. HLA complex comprises region of genes on chromosome 6, which encode HLA antigens involved in various immune response reactions.
- **Genetics of hereditary diseases**. Identification of genetic markers of diseases for which the main etiological factor is genetic, chromosomal or genomic mutations.
- **Reproductive genetics**. The specific feature of this area of diagnosis is examining a male/female couple, who plans to have children.
- **Genetics of multifactorial disorders.** Multifactorial diseases (diseases with hereditary predisposition) develop as a result of interaction of certain combinations of alleles of different loci and specific effects of environmental factors.
- **Pharmacogenetics**. It is the section of medical genetics and pharmacology, studying the dependence of body reactions to drugs upon hereditary factors.

12.1. HLA genotyping kits

The human leukocyte antigen (HLA) is a human histocompatibility gene system – a group of histocompatibility antigens. There are over 150 antigens. The locus is located on chromosome 6; it contains a large number of genes associated with the human immune system (Fig. 15).

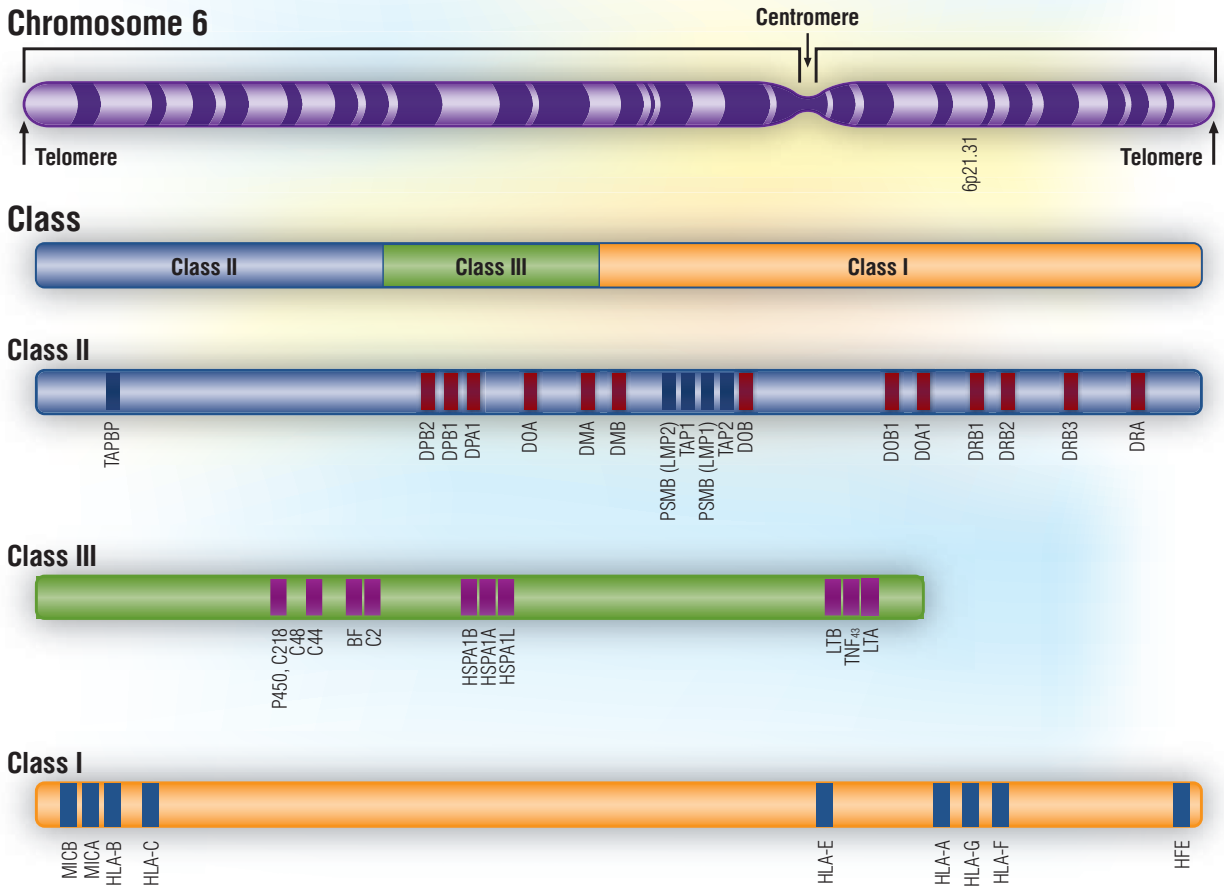


Fig. 15. Major histocompatibility complex (HLA)

MHC class I molecules (A, B, C) are presented on the surfaces of all cell types except for erythrocytes and trophoblast cells.

MHC class II molecules (DP, DM, DQA, DQB, DQ, DR) are presented on the surface of antigen-presenting cells (dendritic cells, macrophages, B-lymphocytes).

MHC class III molecules encode the components of the complement system and proteins, which are presented in the blood.

HLA typing is widely used in the following areas of medicine: identification of tissue compatibility of a donor and recipient in organ and tissue transplantation, differential diagnosis and prognosis of autoimmune diseases, diagnosis of the agnogenic reproductive disorders causes.

For diagnostic purposes HLA typing of the DRB1 gene at low resolution or at the level of 13 allele groups are the most commonly used. In some cases, it is also necessary to type HLA-DQA1 and HLA-DQB1 genes. In most cases, the required level of typing is the allele group: 8 groups for the DQA1 gene and 12 groups for the DQB1 gene.

Organ and tissue transplantation

Genotyping the DRB1 locus at allele group level (low resolution) is used for selecting a tissue-compatible donor and recipient for the first-set grafting of organs, for instance kidneys. This level of genotyping is sufficient and meets the standards of the advanced transplantology centers throughout the world.

Genotyping of DRB1 and DQB1 loci at allele group level is used for selecting a potential donor for familial hematopoietic stem cell transplantation as well as for primary screening of a potential donor in non-familial hematopoietic stem cell transplantation.

Autoimmune diseases

The risk of developing type 1 diabetes, which is the one of the most severe autoimmune diseases, is 10 times higher when either variant from the following genotype is determined: DRB1*01, *03,*04, *08, *09, *10. Taking into account the numerous data from the world literature, the conclusion, which was made in the study of HLA associations with type 1 diabetes can be extended to cover all autoimmune diseases.

Reproductive problems

The difference in a spouses in terms of HLA gene variants is one of the important conditions for successful beginning of pregnancy and carrying of pregnancy. The similarity in a spouses in terms of HLA gene variants increases the likelihood of emergence of an embryo with a double set of identical gene variants, i.e. HLA homozygote, which is an unfavorable factor that may lead to reproductive losses. Therefore, HLA typing of a spouses is used to diagnose causes of reproductive failures to identify similarities in the couple's HLA gene variants.

12.1.1. HLA class II genotyping PCR Kits

DNA-Technology offers the following kits (see Table 24) for HLA class II genotyping by PCR method.

Table 24. HLA class II genotyping PCR Kits

Name	Alleles	Forez	Flash	Rt	qPCR	Registration*
HLA-DRB1 alleles genotyping Kit	DRB1*01, *03, *04, *07, *08, *09, *10, *11, *12, *13, *14, *15, *16)	–	–	*	–	RU/IVD
HLA-DQA1 alleles genotyping Kit	DQA1*0201 and DQA1*0101, *0102, *0103, *0301, *0401, *0501, *0601	–	–	*	–	RU/IVD
HLA-DQB1 alleles genotyping Kit	DQB1*02, *0301, *0302, *0303, *0304, *0305, 0401/0402, *0501, 0502/0504, *0503, *0601, *0602-8	–	–	*	–	RU/IVD
DRB1, DQA1, DQB1 alleles genotyping	DRB1*01, *03, *04, *07, *08, *09, *10, *11, *12, *13, *14, *15, *16; DQA1*0201 and DQA1*0101, *0102, *0103, *0301, *0401, *0501, *0601; DQB1*02, *0301, *0302, *0303, *0304, *0305, 0401/0402, *0501, 0502/0504, *0503, *0601, *0602-8	–	–	*	–	RU/IVD

*** Note:**

RU/IVD – kits for *In Vitro* Diagnostic, which are registered in Russia only

CE/IVD – kits for *In Vitro* Diagnostic, which are registered in EU

RUO – kits for Research Use Only

Kit format:

- Rt:
  – Not pre-aliquoted in tubes – HLA-DQ A1, HLA-DQ B1;
  – Strip tubes (8 pcs., 0.2 ml each) – HLA-DR B1.

Storage temperature:

+2 to +8 °C (Taq-AT-polymerase at -20 °C for kits HLA DQ A1, HLA DQ B1).

Shelf life:

- Rt – 12 months.

DNA extraction kits:

- *PREP-RAPID GENETICS*;
- *PREP-GS GENETICS.*

Specimen for screening:

Whole peripheral blood.

Recommended additional reagents:

- Internal control (IC) is designed for DNA identification (qualitative analysis) and approximate estimation of the amount of human genomic DNA (quantitative analysis) in a human biomaterial by real-time PCR;
- For HLA-DR B1 IC *is included in the kit*.

The following equipment and supplies are required for the analysis:

- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

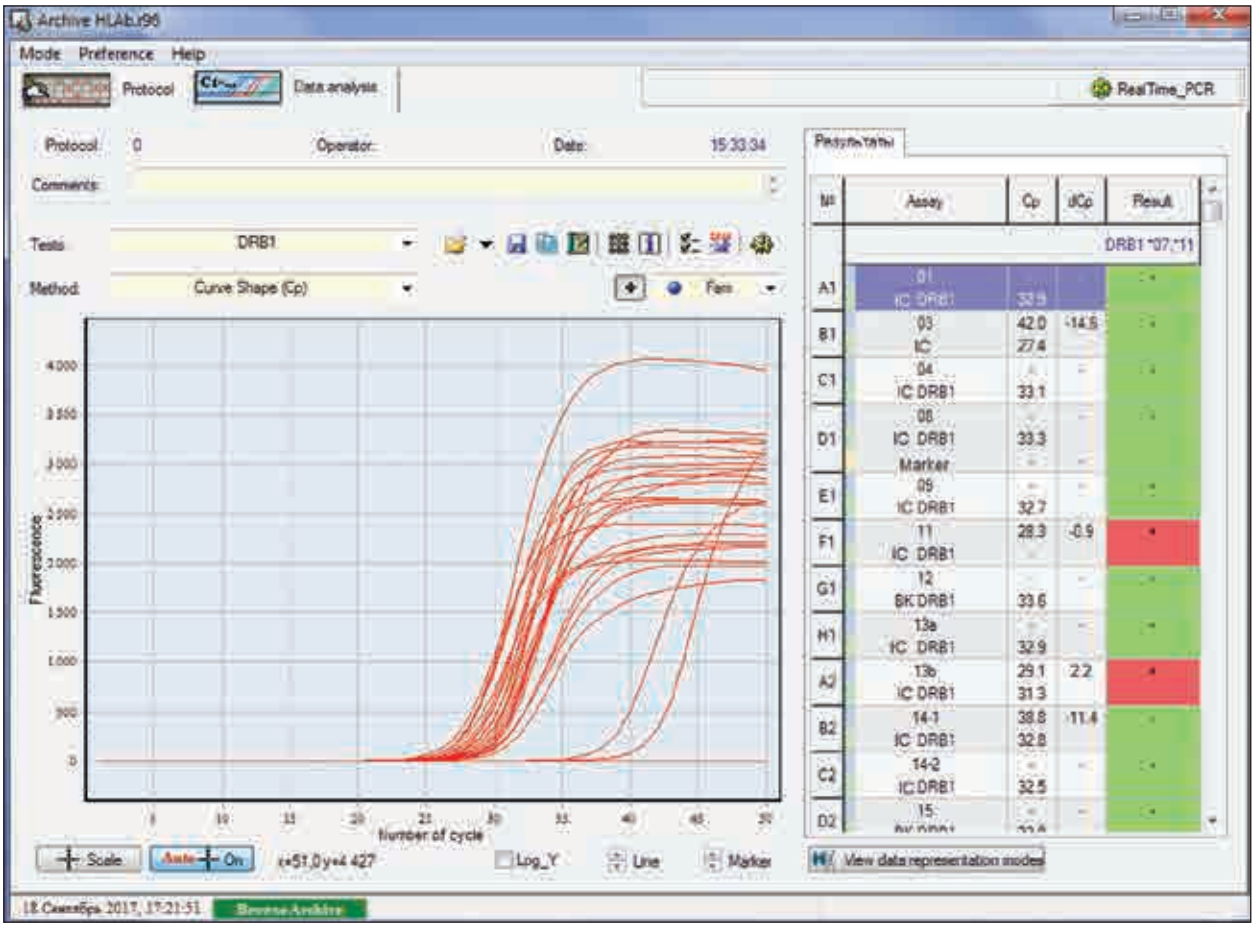
Equipment required for analysis:

- DT devices produced by DNA-Technology (DTlite, DTprime, DT-96)

Software:

Reaction results are analyzed and interpreted automatically (for DT devices produced by DNA-Technology) (Fig.16).

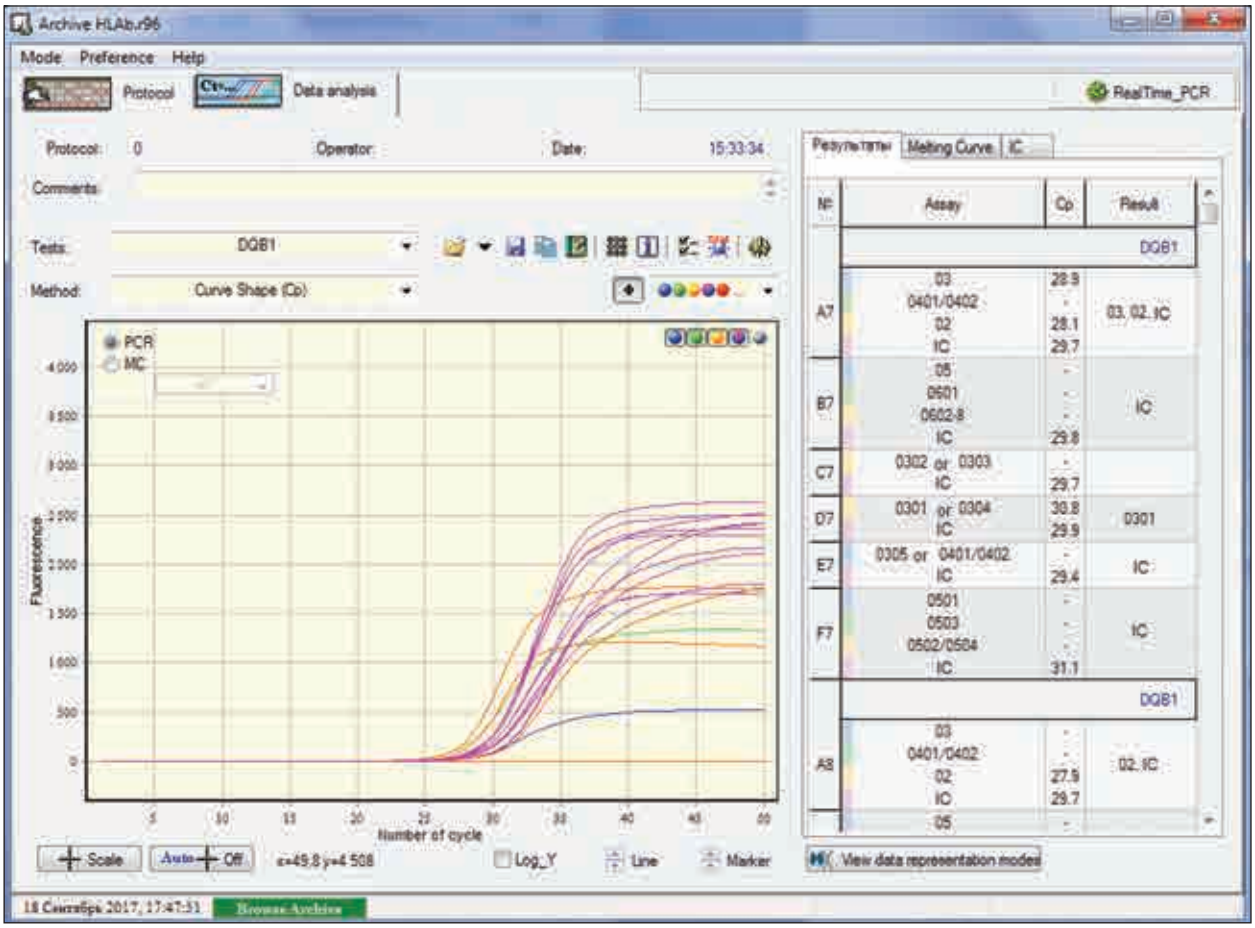
A



B

№	Name of research	Results
1	DRB1	*08, *13

C



D

№	Name of research	Results
1	HLA_DRB1	*08, *13
2	HLA_DQA1	*0301, *0501
3	HLA_DQB1	*20, *0302

Fig. 16. Analysis results for Rt optical measurements (DT devices)

HLA class II genotyping PCR Kits. HLA-DRB1 alleles genotyping Kit

A – Optical measurement analysis

B – Data readout form

HLA class II genotyping PCR Kits. HLA-DQB1 alleles genotyping Kit

C – Optical measurement analysis

D – Data readout form

12.1.2. HLA-B27 alleles genotyping Kit

The genetically caused seronegativity of some diseases make them inaccessible for analysis by standard serological methods, while PCR method gives a positive result, for instance, seronegative spondyloarthritis (SpA) – the disease is characterized by lesions of the sacroiliac joint with tendency to family aggregation. The SpA group includes 10 diseases associated with the presence of HLA-B27 – an antigen: idiopathic ankylosing spondylitis, psoriatic arthritis, Reiter's syndrome, ulcerative colitis, Crohn's disease, Whipple's disease, juvenile chronic arthritis, reactive arthritis (Yersinia, Shigella, and Salmonella), acute anterior uveitis and Behcet's syndrome.

HLA-B27 carriage detection is one of the most advanced approaches in early diagnosis and selection of treatment for a number of autoimmune diseases.

According to International classification of rheumatic diseases, there is a separate group of spondyloarthritis associated with the HLA-B27 antigen (see Table 25).

Table 25. Group of diseases associated with HLA-B27 antigen

Disease	HLA-B27 incidence (%)
Ankylosing spondylitis	90-95
Reiter's disease	70-85
Reactive arthritis	36-100
Psoriatic arthritis	54
Enteropathic arthritis	50

These diseases are often accompanied by serious complications such as peripheral arthritis, eye disease, and lesions of the urinary tract, intestines and skin. There is an estimated 20-30% risk of developing disease in an individual who is a carrier of HLA-B27.

Indications for genetic HLA-B27 test:

- Necessity to eliminate a possibility of ankylosing spondylitis in a patient, whose relatives are suffering from this disease;
- Differential diagnosis of incomplete forms of Reiter's syndrome (without urethritis or uveitis) with gonococcal arthritis;
- Differential diagnosis of Reiter's syndrome, accompanied by severe arthritis and rheumatoid arthritis;
- When examining patients with juvenile rheumatoid arthritis. If the HLA-B27 antigen is not detected, ankylosing spondylitis and Reiter's syndrome are unlikely, although these diseases cannot be completely excluded in this case.

Recommended additional studies:

- X-ray examination of the sacral region of the spine;
- Examinations for detection tuberculosis infection, toxoplasmosis infection, herpesvirus infection, chlamydial infection, *Klebsiella pneumoniae, Salmonella, Yersinia, Shigella* and other bacterial infections.

DNA-Technology offers a kit (see Table 26) for HLA class I genotyping by PCR method.

Table 26. HLA-B27 alleles genotyping Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
HLA B27 alleles genotyping Kit	–	–	*	–	RU/IVD

*** Note:**

RU/IVD – kits for *In Vitro* Diagnostic, which are registered in Russia only

CE/IVD – kits for *In Vitro* Diagnostic, which are registered in EU

RUO – kits for Research Use Only

Kit format: Strip tubes (8 pcs., 0.2 ml each).

Storage temperature: +2 to +8 °C.

Shelf life: Rt – 12 months.

DNA extraction kits:

- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Specimen for screening: Whole peripheral blood.

Recommended additional reagents:

- *Sample intake control* (SIC) is designed for DNA identification (qualitative analysis) and approximate estimation of the amount of human genomic DNA (quantitative analysis) in a human biomaterial by real-time PCR.

The following equipment and supplies are required for the analysis:

Strip plastic rack and centrifuge (vortex) rotor.

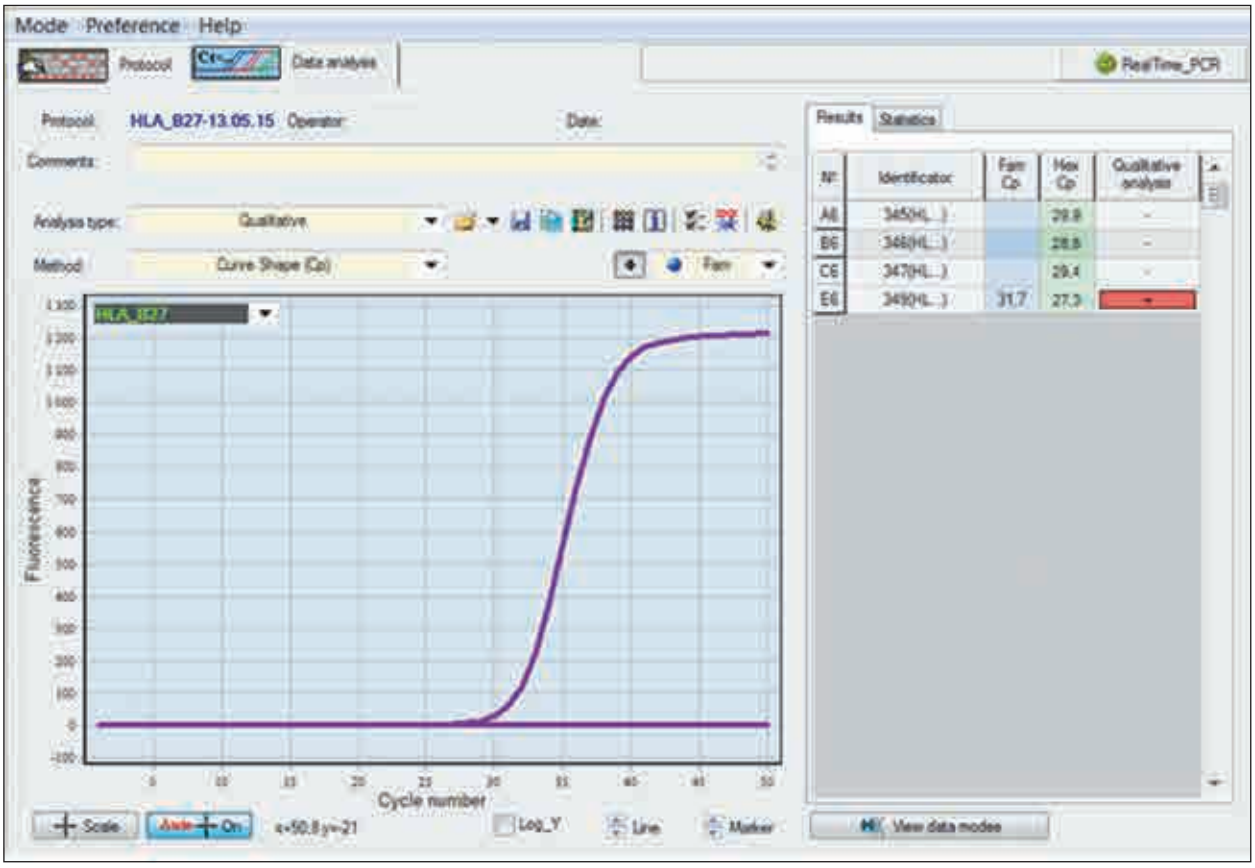
Equipment required for analysis:

DT devices produced by DNA-Technology (DTlite, DT-prime, DT-96).

Software:

Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 17).

A



B Qualitative analysis

Number of the hole	Identificator of the tube	Cp, Fam	Cp, Hex	Result
A6	345 (HLA B27)		29,9	–
B6	346 (HLA B27)		28,6	–
C6	347 (HLA B27)		29,4	–
E6	349 (HLA B27)	31,7	27,3	+

Fig. 17. Optical measurement analysis results

A – Optical measurement analysis (Fam channel)

B – Analysis report

12.2. Rare inherited diseases

It is necessary to distinguish the concept of "**hereditary**" and "**congenital**" diseases. Congenital diseases are already evident at birth, and they may be hereditary. Hereditary diseases are result from mutations in the genetic apparatus of the cell. Where in, **mutation is one and sufficient cause for the development of hereditary disease**. If a person has inherited a mutation, he is either sick or asymptomatic carrier, **which allows to make or verify a diagnosis based on detection of the relevant genetic marker**[4].

Hereditary diseases are chronic in nature, caused by the presence of a mutant gene; they manifest themselves throughout a person's life and have a relapsed or pro-gradient (progressive) clinical course. In chronic pathological process, in-depth genetic test is recommended to identify the molecular causes of the disease and make etiologically based diagnosis.

Mutations are divided into genomic, chromosomal and gene mutations based on the size of damage to the genetic material. Hereditary diseases are classified likewise. Gene diseases represent the largest group.

Monogenic genetic disorders are hereditary diseases caused by mutations in a single gene. There are currently over 6,500 known mutations in 3,500 loci that cause genetic diseases [10]. The aggregate contribution of all monogenic abnormality to overall disease incidence accounts for 1 % of cases on average [14, 23].

PCR-based DNA study is the most accurate, objective and informative method for diagnosing genetic diseases. The presence of a mutant gene can be detected prior to establishing a clinical picture of the disease, which is particularly important for diagnosis, prevention or treatment. In this case, timely diagnosis allows to provide a pathogenetically-based treatment of a hereditary disease that is usually resistant to conventional therapies (for instance use of active vitamin D metabolites in hereditary rickets diseases that are resistant to anti-rickets doses of vitamin D). Moreover, it is possible to prevent a number of serious clinical symptoms, such as mental retardation caused by phenylketonuria or cirrhosis and liver cancer in hemochromatosis, before they develop.

The vast majority of mutations are transmitted from generation to generation, which makes it important to examine not only a patient but also his closest relatives. Possibility to detect the damages of genetic material and knowledge of inheritance type can provide a benefit in assessing a risk of developing the disease in posterity [16].

DNA-Technology offers the following kits (see Table 27) for detecting genetic polymorphisms causing monogenic genetic disorders using real-time PCR method.

Table 27. Kits produced by DNA-technology for detecting genetic polymorphisms causing monogenic genetic disorders using real-time PCR method

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
HEMOCHROMATOSIS	–	–	*	–	CE/IVD
Cystic Fibrosis SCREEN (8 SNPs) REAL-TIME PCR Genotyping Kit	–	–	*	–	RUO
Cystic Fibrosis - rare CFTR mutations (16 SNPs) REAL-TIME PCR Genotyping Kit	–	–	*	–	RUO

*** Note:**

RU/IVD – kits for *In Vitro* Diagnostic, which are registered in Russia only

CE/IVD – kits for *In Vitro* Diagnostic, which are registered in EU

RUO – kits for Research Use Only

12.2.1. HEMOCHROMATOSIS. SNP Genotyping Kit

Hemochromatosis (pigment cirrhosis, bronze diabetes) is a disease characterized by congenital or hereditary disorder of iron metabolism in the human body.

There are two kinds of it:

- *Primary* (classical, hereditary) hemochromatosis;
- *Secondary* hemochromatosis, which is caused by repeated hemolytic and megaloblastic anemia crises, multiple blood transfusions and wrong treatment with iron drugs. Secondary hemochromatosis is not a genetically determined disease.

Hereditary hemochromatosis (HHC) is a genetically determined disease that manifests itself in iron metabolism disorder, its accumulation in a body's tissues and organs: liver, pancreas, myocardium, spleen, skin, endocrine glands and other organs. HHC triggers a number of diseases: cirrhosis and liver cancer, heart failure, diabetes and arthritis.

Hereditary hemochromatosis is classified into four types depending on the genetic basis of the disease:

Type I is inherited in an autosomal recessive way and it is caused by mutations in the HFE gene that is located on chromosome 6.

Type II (juvenile hemochromatosis) is caused by mutations in the gene that is responsible for synthesis of other iron metabolism protein - hepcidin;

Type III is caused by mutations of the gene that encodes synthesis of transferrin receptor.

Type IV is caused by mutations of the gene SLC40A1 that encodes synthesis of the transport protein of the ferroportin.

Type I hemochromatosis is the most common and thus determining the necessity for molecular genetic diagnostics.

Iron supply is regulated by interaction between transferrin receptor (TfR1) with normal HFE gene product, which inhibits transport of transferrin-iron complex through the cell membrane, and thus iron accumulates in the enterocyte as ferritin. In hereditary hemochromatosis type I, the most common are mutations leading to amino acid substitutions in 63, 65 and 282 positions of **HFE** protein: **H63D**, **S65C** and **C282Y**.

Defective HFE cannot modulate expression of hepcidin that normally regulates metabolism of iron and intestinal iron absorption processes. This leads to dysregulation of absorption and distribution of iron in a body and to excessive accumulation in cells.

HHC prevalence varies from 1:250 individuals living in Northern Europe to 1:3300 among African-American population of the United States and African population. During genetic screening was determined that 1 out of the 500 examined persons had homozygous mutations in the HFE gene, while the number of clinically established cases of hereditary hemochromatosis was found to be 1:5000 [23].

This is due to the incomplete penetrance of the disease (not always evident in carriers of the mutations even in a homozygous state) and significant contribution by external factors: use low iron foods and loss of iron from bleeding (typical for women before menopause). Mostly the disease develops because of in iron-rich diet and alcoholism. Women with amenorrhea may also be vulnerable to development of clinically significant symptoms of hemochromatosis.

The most frequent mutation S282Y (is found in 87-90 % of patients) is substitution of cysteine by tyrosine at amino acid position 282. It leads to inability of protein to interact with TFR1, resulting in false signal of a low iron content in the body and increasing its absorption.

The less frequent mutation is N63D, a substitution of cytidine by guanine at amino acid position 65 (approximately 3-5 % of patients), which reduces affinity to TFR1 to a lesser degree (see Table 28).

Table 28. Possible compound heterozygous genotypes associated with hereditary hemochromatosis

Compound heterozygotes	Polymorphisms	Genotypes identified	Clinical manifestations
Variant 1	**845 G>A (C282Y)**	**G/A**	High risk of hereditary hemochromatosis.
	187 C>G (H63D)	**C/G**	Hereditary hemochromatosis type I is diagnosed in iron overload syndrome
Variant 2	**845 G>A (C282Y)**	**G/A**	High risk of hereditary hemochromatosis.
	193 A>T (S65C)	**A/T**	Hereditary hemochromatosis type I is diagnosed in iron overload syndrome
Variant 3	**187 C>G (H63D)**	**C/G**	Higher risk of hereditary hemochromatosis developing.
	193 A>T (S65C)	**A/T**	Hereditary hemochromatosis type I is diagnosed in iron overload syndrome. Severe disease is not typical

The majority of patients with typical HHC phenotype are homozygous for mutant 282Y allele and a smaller part of patients are carriers of 63D/63D homozygote, which usually have not a severe form of the disease. Also, a small percentage of patients are compound heterozygous (282Y/63D).

In S65C homozygous genotype, a mild form of hemochromatosis is formed.

Patients with 282C/282Y genotype have no clinically significant symptoms of hemochromatosis, but they are prone to increased content of ferritin and increased transferrin's saturation levels.

Clinical picture of HHC

Manifestation of the disease is usually detected at the age of 40-60 years and is accompanied by characteristic clinical symptoms. Latent (pre-cirrhotic) stage of the disease can be detected much earlier. Disease is diagnosed in men 5-10 times more frequently than in women. In the initial stage of the disease, there are usually no characteristic clinical symptoms. This case the diagnostic criteria are indicants of iron metabolism laboratory parameters (determination of the level of serum ferritin and others). If the disease is not detected at an early stage, further accumulation of iron would lead to advanced stage of the disease with liver cirrhosis, specific skin color changes (bronze skin) and multiple organ failure. Diabetes would be developed in approximately 65 % of patients, arthropathy – in 25-50 % and heart pathology (including congestive heart failure) – in 15 %. Almost 30 % of patients develop liver cancer, which is the most common cause of death [22].

HHC diagnostics

Hemochromatosis diagnosis is based on the presence of familial cases of the disease, elevated levels of serum iron and multiple organ damage.

Molecular genetic diagnosis of HHC allows making a diagnosis of HHC without using additional complex and costly methods. Laboratory parameters should be measured over time and cannot substitute molecular genetic diagnosis, because there is no iron overload syndrome in the latent stage of hemochromatosis.

If existence of iron overload syndrome is proved by the laboratory and if the patient is homozygous for C282Y or compound heterozygous for C282Y/H63D, HHC diagnosis can be considered established. For verification of the diagnosis in case of confirmed carriage of these mutations and in the absence of clinical symptoms of cirrhosis, there is no need to carry out liver biopsy.

Timely initiated treatment (bloodletting and use of iron chelators) and complex preventive measures (diet with reduced iron content and limiting alcohol consumption) can completely prevent the development or further progression of the disease [135].

Indications for genetic analysis:

- Diagnosis of the presence of clinical picture of hemochromatosis;
- Forecast of disease severity;
- Pre-symptomatic diagnosis and prevention of the disease;
- Testing of direct relatives of patients with verified forms of hereditary hemochromatosis.

DNA-Technology developed a kit (see Tables 28 and 29) for identification of polymorphisms associated with hemochromatosis using real-time PCR method.

Table 28. Genetic polymorphisms associated with hemochromatosis

Gene	Polymorphism	Identifier*	Genotype	Clinical manifestations
HFE – gene encoding a hereditary hemochromatosis protein	**845 G>A (C282Y)**	**rs1800562**	**G/G**	No abnormalities
			G/A	Tendency to increase ferritin content and transferrin saturation percentage
			A/A	High risk of hereditary hemochromatosis. Hereditary hemochromatosis type I is diagnosed in iron overload syndrome
	187 C>G (H63D)	**rs1799945**	**C/C**	No abnormalities
			C/G	Asymptomatic carriage
			G/G	Higher risk of hereditary hemochromatosis developing. Hereditary hemochromatosis type I is diagnosed in iron overload syndrome. Severe disease is not typical
	193 A>T (S65C)	**rs1800730**	**A/A**	No abnormalities
			A/T	Asymptomatic carriage
			T/T	Higher risk of hereditary hemochromatosis developing. Hereditary hemochromatosis type I is diagnosed in iron overload syndrome. Severe disease is not typical

** Labeling in the dbSNP database of the National Center for Biotechnological Information, NCBI*

Table 29. HEMOCHROMATOSIS. SNP Genotyping Kit

Number of tests	48 tests
Kit format	not pre-aliquoted
Taq-AT polymerase	1 tube (72 mcl)
PCR buffer	1 vial (1,44 ml)
Mineral oil	1 vial (2,88 ml)
Polymorphisms to be identified	HFE: 187 C>G (H63D) – 960 mcl HFE: 193 A>T (S65C) – 960 mcl HFE: 845 G>A (C282Y) – 960 mcl
Specimen for analysis	Whole blood
Shelf life	6 months

Technology:
- PCR melting;
- Use of other technological platforms is not permitted;

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, DT-prime, DT-96.

Shelf life: 6 months.

Storage temperature:
+2 to +8 °C (-20°C for Taq-AT-polymerase).

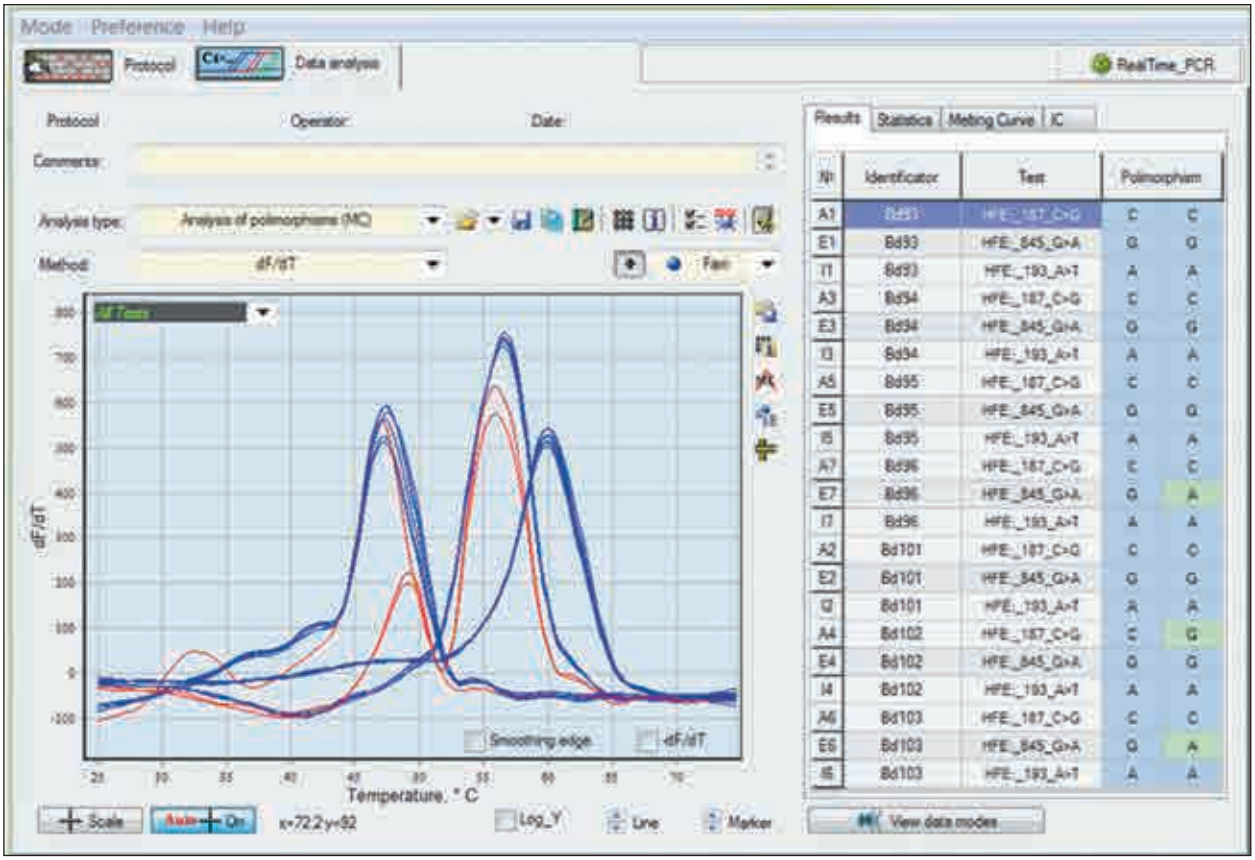
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 18).

A



B

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

Fig. 18. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.2.2. Cystic Fibrosis REAL-TIME PCR Genotyping Kit

Cystic fibrosis (CF), also known as mucoviscidosis is an inherited disease (OMIM: 219700) associated with ion transport disorder in the epithelium and caused by mutations in the CFTR gene (Cystic fibrosis transmembrane conductance regulator). The protein, which is encoded by this gene, functions as a cAMP-dependent chloride channel, that is embedded in the cell membrane. In order for the disease is developed, there must be damage to both alleles (inherited both from the mother and from the father) – autosomal recessive inheritance. CF frequency varies depending on the population, but, on average, it is 1 from 2500-4500 Europeans born with the disease.

Cystic fibrosis is a serious systemic disease, accompanied by significant decline in the quality and duration of life. Patients with CF could suffer multiple organ failure, especially for organs that have mucous secretion: upper and lower respiratory tract, pancreas, biliary system, intestine, male genital and sweat glands. Some patients have meconium ileus at birth. In hot climates, as well as in infants with not-yet-fully developed thermal control and increased release of electrolytes in sweat, life-threatening condition may develop – Pseudo-Bartter's syndrome (symptom complex that manifests itself in the form of hypochloremia, metabolic alkalosis and dehydration) [61, 71].

Classification of CFTR gene mutations

Depending on the degree of the protein damage, CFTR gene mutations are divided into classes (see Table 30). The most damaged proteins are referred to the class I–III mutations. They lead to synthesis of the truncated protein, its defective maturation (folding) or defective chloride channel response to cAMP stimulation. The degree of protein damage determines the severity of phenotypic manifestations of the disease. Class I–III mutations are phenotypically severe mutations. Cases of cystic fibrosis caused by these mutations are more often severe in nature and are characterized by early onset of serious complications and exocrine pancreatic insufficiency. Identification of such mutations could be a basis for correction of patients' management tactics.

Table 30. Classes of CFTR gene mutations and their phenotypes (Zielenski &Tsui, 1995, Green et al, 2010).

Class	Degree of protein damage	Phenotypes	Main mutations
Class I	**Protein synthesis with changed primary structure** Gene mutations (frameshift due to insertions, deletions and nonsense mutations) lead to critical reduction in number of chloride channels on the cell surface or to their total absence. This may be due to defective mRNAs (unstable form), defective mRNA splicing and/or defective synthesis of the protein's amino acid sequence. It leads to formation of an unstable protein structure, which is again degraded in the cell cytoplasm, or truncated and nonfunctional form. The latter is associated with premature termination of stop codon translation	Mostly severe	G542X, 1078delT, 1154 insTC, 1525-2A>G, 1677delTA, 1717-1G>A, 1898+1G>A, 2143delT, 2184 delA, 2184 insA, 3007 delG, 3120+1 G>A, 3659 delC, 3876 delA, 3905insT, 394 delTT, 4010 delA, 4016 insT, 4326 delTC, 4374+1 G>T, 441 delA, 556 delA, 621+1G>T, 621-1 G>T, 711+1 G>T, 875+1 G>T, 875+1 G>C, E1104X, E585X, E60X, E822X, G542X, G551D/R553X, Q493X, Q552X, Q814X, R1066C, R1162X, R553X, V520F, W1282X, Y1092X
Class II	**Defective protein maturation (formation of its secondary and tertiary structures). The protein does not reach the cell membrane and it is destroyed in the cytoplasm** CFTR mutations lead to formation of the small defective channels or to complete absence on the cell surface due to defective protein processing and transportation	Mostly severe	A559T, D979A, F508del, I507del, G480C, G85E, N1303K, S549I, S549N, S549R
Class III	**Defective chloride channel response on cAMP stimulation.** Normal amount of non-functional protein is formed in the cell membrane	Mostly severe	G1244E, G1349D, G551D, H199R, I1072T, I48T, L1077P, R560T, S1255P, R75Q
Class IV	**Reduction of the chlorine channel conductibility** CFTR mutations cause formation of protein with normal response on cAMP stimulation, but with low amplitude of ion current and shorter residence time of the channel in open state.	Variable / mild	A800G, D1152H, D1154G, D614G, delM1140, E822K, G314E, G576A, G622D, G85E, H620Q, I1139V, I1234V, L1335P, M1137V, P67L, R117C, R117H, R334W, R347H, P347P, R792G, S1251N, V232D
Class V	**Reduction of functionally active protein amount, disorder of protein transportation to the cell membrane** CFTR mutations lead to a reduction in the amount of mRNA and defective translation process. As a consequence, insufficient amount of chloride channels for regulation of ion homeostasis is formed.	Mostly mild	2789+5 G>A, 3120 G>A, 3272-26 A>G, 3849+10kbC>T, 621+3 A>G, 711+3 A>G, A445E, IVS 8 poly T, P574H

Male infertility associated with CFTR mutations

Men with mucoviscidosis (cystic fibrosis) almost always have azoospermia and infertility due to bilateral aplasia of vas deferens (OMIM: 277180 – congenital bilateral absence of vas deferens, CBAVD).

In some cases, men are diagnosed with genital form of cystic fibrosis that leads to infertility with almost total absence of clinical manifestations of cystic fibrosis (CF) or existence of minimal CF clinical manifestations.

It is believed that up to 70 % of men, who are suffering from infertility and azoospermia (absence of sperm in the semen) or severe oligozoospermia, are carriers of CFTR gene mutations. Wherein, the mutations are often detected only in one allele.

Absence of vas deferens is often not detected clinically. Therefore, all patients with azoospermia, especially those whose sperm count is less than 1.5 million and pH<7, are advised to undergo screening for CFTR mutation carrier in order to exclude CBAVD.

For genetic causes of infertility, treating patients with conservative methods (hormone therapy) is ineffective, but application of IVF and ICSI allowes these patients to have their own children. Wherein, taking into account the high population frequency of carriage of CFTR mutations, a risk of birth a child with CF is significantly high in such a situ-

ation. In this regard, genetic diagnosis of CFTR mutation carriage in women is necessary. If a mutation is detected in the woman, the couple should schedule preimplantation or prenatal genetic diagnosis, because there is a high probability (25 %) of having a sick child. Genetic diagnosis may also be recommended for the patient's close relatives [24].

The conference of the National Institute (USA) in 1997 recommended that all married couples who plan pregnancy, even those without a family history of CF, should be screened for CFTR gene mutations.

Cystic fibrosis diagnosis

Genetic diagnosis is important for cystic fibrosis diagnosis in newborns, including when it is not possible to undergo sweat test (small amount of sweat, low weight of the child). Identification of CFTR gene mutations makes it possible to make a diagnosis and initiate treatment at the pre-clinical stage.

Pathogenetic therapy in cystic fibrosis, so-called "correctors" and "potentiators", are widely being developed [113]. Identifying specific mutations in a patient allows, in some cases, to choose a particular drug when prescribing such therapy.

Moreover, frequency of certain mutations varies depending on the population (see Table 31, Fig. 19).

Table 31. CFTR gene mutations with high frequency (10% and above) in some populations (excluding ΔF508 mutation) [29]

Mutation	Population	Frequency (%)
Q359K/T360K	Georgian Jews	88
M1101K	Hutterites	69
S549K	United Arab Emirates	61.5
W1282X	Ashkenazi Jews	48
	Tunisian Jews	17
	Israeli Arabs	10.6
405+1 G>A	Tunisian Jews	48
	Libyan Jews	18
3120+1 G>A	Bantu, Africa	46.4
	South Africa	17.4
	African-Americans, US	31.9
	African-Americans, Africa	12.2
	Saudi Arabia	10
N1303K	Egyptian Jews	33
	Israeli Arabs	21
	Algeria	20
	Lebanon	10
G85E	Turkish Jews	30
1898+5 G>T	Taiwan	30
394delTT	Finland	28.8
	Estonia	13.3
621+1 G>T	Saguenay, Canada	24.3
	Northern Greece	12.1

Mutation	Population	Frequency (%)
Y122X	Reunion, East Africa	24
3905 insT	Amish, Mennonites	16.7
	Switzerland	9.8
Y569D	Pakistan	15.4
T338I	Sardinia, Italy	15.1
1548delG	Saudi Arabia	15
R553X	Switzerland	14
3120+1kb del8.6kb	Israeli Arabs	13
I1234V	Saudi Arabia	13
R347P	Bulgarian Turks	11.7
Q98X	Pakistan	11.5
G542X	Southern Spain	11.4
711+1 G>T	Algeria	10
4010del4	Lebanon	10
R1162X	Northeast Italy	9.8
1525-1 G>A	Pakistan	9.6

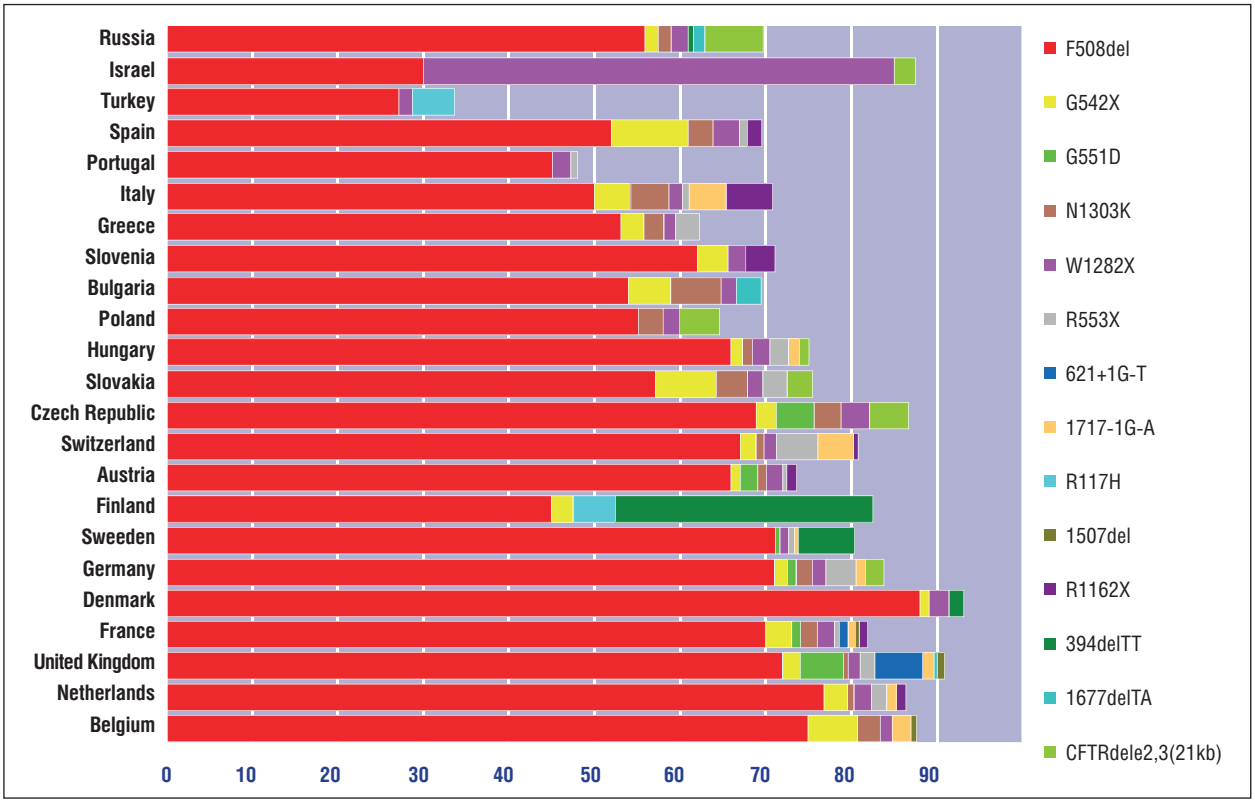


Fig. 19. Relative frequency of CFTR mutations in some populations of Eurasia (Petrova N.V., 2010)

According to WHO, the F508del CFTR mutation is the most common mutation causing CF. *A register of identified mutations based on ethnic groups is published in the WHO report "The molecular genetic epidemiology of cystic fibrosis"*, published in 2004.

According to the report, in Europe, F508del has a frequency of about 70 % (frequencies vary from a maximum of 100 % in the isolated Faroe Islands of Denmark, to a minimum of about 20 % in Turkey). Among other CFTR gene mutations in Europeans, the relatively frequent mutations are G542X, N1303K, and G551D. Among individual ethnic groups, you have the Swiss with high frequency of 3905insT mutation, 394delTT mutation in the Norwegians, R1162X mutation in Northeast Italy, and Eastern Slavic CFTRdele2,3 (21kb) mutation.

There is a similar picture concerning the studies of the spectrum of CFTR gene mutations in Africa, where a high degree of similarity in Algeria and Tunisia with European mutations is registered, (prevalence of F508del, G542X and N1303K). On the contrary, in southern Africa, the second most prevalent allele is the 3120+1G→A mutation (up to 46 % of the total number of identified CFTR mutations). The same trend was observed in African Americans in North America, whereas F508del (58 %), 621+1G→T (23 %) and A455E (8 %) predominate in Canada. In Latin America, the F508del mutation is also the most frequent CF-causing CFTR mutation (e.g. 59 % in Argentina, but only 29 % in Chile). In the Middle East, the Arab population is dominated by F508del, N1303K, W1282X and 3120+1G>A. Given the diversity of ethnic groups in the territory, the spectrum of mutations also includes: CFTRdele2 (ins186) in Muslim Arabs, 4010delTAAT in Christian Arabs, S549R(T>G) mutation in Bedouins from the United Arab Emirates, and the 1548delG mutation in Saudi Arabia.

Investigation of diagnostically significant CFTR mutations in Russian CF patients found that F508del, CFTRdele2,3 (21kb), 2143delT, W1282X, N1303K, 3849+10kbC-T, 2184insA, G542X, 1677delTA, 3821delT, R334W, L138ins and 394delTT account for at least 60-75 % in different regions. In this case, among the ethnic Russians, the most frequent mutation is F508del (54.4 %) and followed by CFTRdele2,3 (21kb) (6.6 %). F508del mutation in patients belonging to other ethnic groups is less common than in the ethnic Russians: Tatars – 44 %, Chechens – 30 %, and Georgians – 17 %. The W1282X mutation occurs most frequently in Jewish patients or those from mixed marriages between Jews and other ethnic groups [17]. The 1677delTA mutation is often found in indigenous people of the Caucasus and Transcaucasia (Georgians, Chechens), while G542X mutation is observed in Armenian patients [17].

So, the world practice of CF diagnosis has established an approach to molecular genetic test for the presence of CFTR gene mutations. In the United States in 2005, the FDA recommended molecular diagnostic tests for diagnosis of cystic fibrosis [90].

It should be noted that non-detection of mutations does not exclude CF diagnosis, since a patient can have extremely rare mutations that cannot be identified by use of a panel.

For improvement in the economic efficiency of diagnostics, a two-step diagnostic algorithm is recommended [38]:

- **Level 1:** Analysis for the presence of frequent mutations, which can achieve over 70 % detection
- **Level 2:** Study of rarer mutations, which can achieve 85 % detection

Indications for genetic analysis:

- In the complex of diagnostic methods for establishing or verifying CF diagnosis;
- For pre-implantation and prenatal CF diagnosis in known mutations in the proband and proband's parents;
- Given high frequency of carriage of CFTR gene mutations, it is recommended to go for detection of carriage in relatives of patients (first and second degree relatives) and their husbands/wives in planned pregnancy;
- As part of a package of measures during planning of pregnancy, especially in consanguineous marriages;
- In the complex of diagnostic methods to establish the causes of infertility in men (especially in bilateral or unilateral aplasia of the vas deferens and/or obstructive azoospermia);
- When deciding on the use of assisted reproductive technologies to overcome infertility;

In view of these recommendations, DNA-Technology has developed a kit (see Tables 32 and 33) for detecting CFTR gene mutations associated with cystic fibrosis using real-time PCR method.

Table 32. CFTR mutations associated with cystic fibrosis, detected using real-time PCR method – CYSTIC FIBROSIS SCREEN (8 SNPs)

Gene	Gene function	Polymorphism	Identifier*	Genotype	Clinical manifestations
CFTR – (cystic fibrosis transmembrane conductance regulator)	Encodes a protein that functions as a cAMP-dependent chloride channel	F508del	rs113993960	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class II**
		dele2,3 (21kb)		**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class I**
		2143delT	rs121908812	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class I**
		W1282X	rs77010898	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class I**
		N1303K	rs80034486	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class II**
		3849+10kbC>T	rs75039782	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class V**
		1677delTA	rs121908776	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class I**
		E92K	rs121908751	**NN**	No abnormalities
				Nm	
				mm	Degree of protein damage matches with **class II**

** Identification in dbSNP National Center for Biotechnological Information, NCBI (USA).*

Table 33. CFTR mutations associated with cystic fibrosis, detected using real-time PCR method – CYSTIC FIBROSIS - rare CFTR mutations (16 SNPs)

Gene	Gene function	Polymorphism	Identifier*	Genotype	Clinical manifestations
CFTR – Cystic fibrosis transmembrane conductance regulator	The protein encoded by this gene functions as a cAMP-dependent chloride channel	2184insA	rs121908786	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		3821delT	rs77035409	**NN**	No abnormalities
				Nm	Degree of protein damage does not correspond to any class of present classification
				mm	
		R334W	rs121909011	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class IV**
				mm	
		L138ins	_	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class IV**
				mm	
		394delTT	rs121908769	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		S1196X	rs121908763	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		604insA	_	**NN**	No abnormalities
				Nm	Degree of protein damage is predominantly **severe**
				mm	
		3944delTG	rs78756941	**NN**	No abnormalities
				Nm	Degree of protein damage is predominantly **severe**
				mm	

Gene	Gene function	Polymorphism	Identifier*	Genotype	Clinical manifestations
		621+1G-T	_	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		2183AA>G	rs121908799	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		2789+5G>A	rs80224560	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class V**
				mm	
		R117H	rs78655421	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class IV**
				mm	
		R553X	rs74597325	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	
		K598ins	_	**NN**	No abnormalities
				Nm	Degree of protein damage is predominantly **mild**
				mm	
		3667insTCAA	_	**NN**	No abnormalities
				Nm	Degree of protein damage is predominantly **severe**
				mm	
		G542X	rs113993959	**NN**	No abnormalities
				Nm	Degree of protein damage corresponds to **class I**
				mm	

** Identification in dbSNP National Center for Biotechnological Information, NCBI (USA).*

Table 34. Cystic Fibrosis REAL-TIME PCR Genotyping Kits

Number of tests	48 tests
Kit format	not pre-aliquoted
Taq-AT polymerase	1 tube (192 mcl)
PCR buffer	1 vial (3,84 ml)
Mineral oil	1 vial (7.86 ml)
Polymorphisms to be identified	CFTR: F508del – 960 mcl CFTR: G542X – 960 mcl CFTR: W1282X – 960 mcl CFTR: N1303K – 960 mcl CFTR: 2143delT – 960 mcl CFTR: 2184insA – 960 mcl 3849+10kb C>T – 960 mcl dele2,3 (21kb) – 960 mcl CFTR:2184insA – 960 mcl CFTR:3821delT – 960 mcl CFTR:R334W – 960 mcl CFTR:L138ins – 960 mcl CFTR:394delTT – 960 mcl CFTR:S1196X – 960 mcl CFTR:604insA – 960 mcl CFTR:3944delTG – 960 mcl CFTR:621+1G-T – 960 mcl CFTR:2183AA>G – 960 mcl CFTR:2789+5G>A – 960 mcl CFTR:R117H – 960 mcl CFTR:R553X – 960 mcl CFTR:K598ins – 960 mcl CFTR:3667insTCAA – 960 mcl CFTR:G542X – 960 mcl
Specimen for analysis	Whole blood
Shelf life	6 months

Technology:
- PCR melting;
- Use of other technological platforms is not permitted

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, dt-prime, DT-96.
Shelf life: 6 months.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

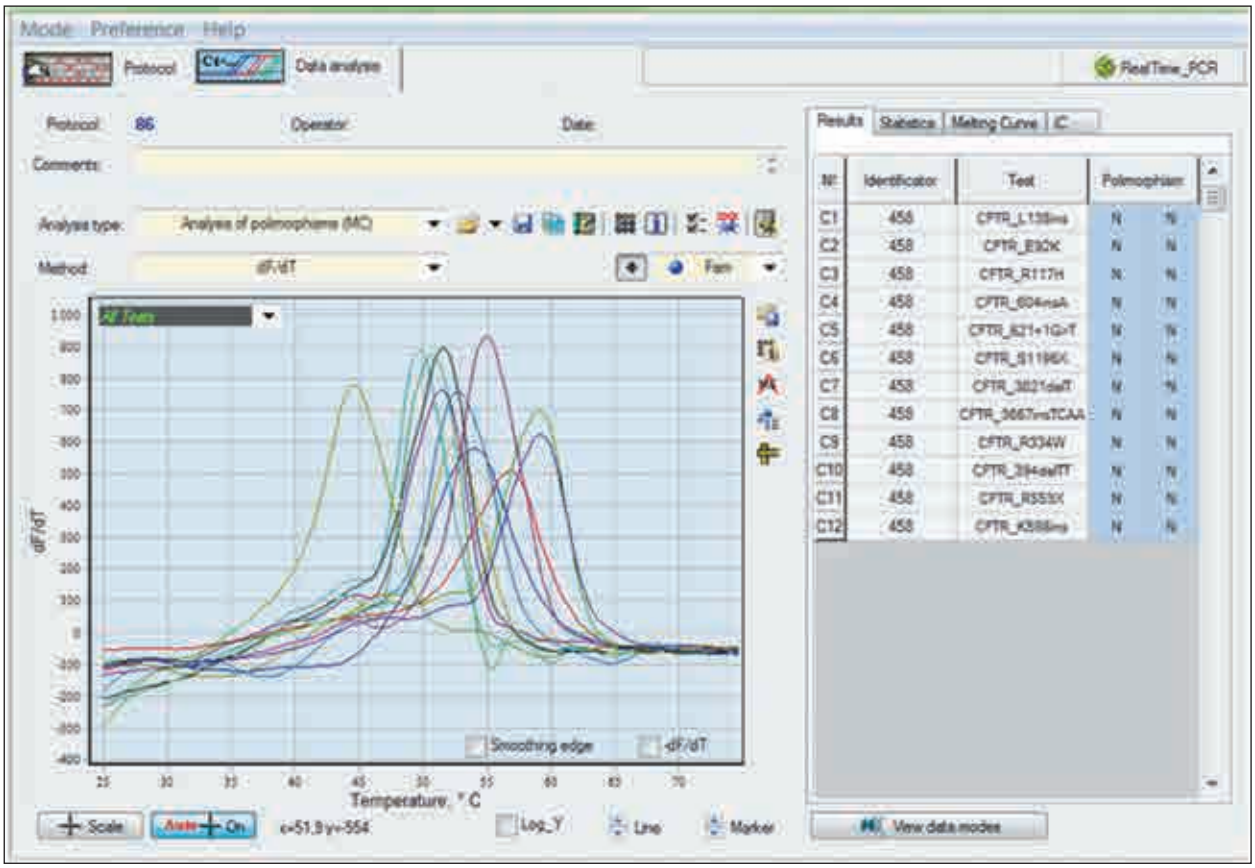
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for the use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 20).

A



B

№	Name of research	Results
		Genotype
1	CFTR_F508del	N N
2	CFTR_E92K	N N
3	CFTR_W1282X	N N
4	CFTR_N1303K	N N
5	CFTR_2143delT	N N
6	CFTR_1677delTA	N N
7	CFTR_3849+10kbC>T	N N
8	CFTR_dele2,3 (21kb)	N N

Fig. 20. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.2.3. Phenylketonuria Screen REAL-TIME PCR Genotyping Kit for detection of the 4 most common genetic polymorphisms of PAH gene associated with inherited risk of phenylketonuria

Phenylketonuria – is a hereditary disease associated with a violation of the metabolism of amino acids, mainly phenylalanine. Without proper therapy **Phenylketonuria** is accompanied by the accumulation of phenylalanine and its toxic products in the body, which leads to severe damage to the central nervous system and impaired mental development. The frequency and prevalence of the disease varies depending on the population. The highest birth rate of a child with PKU was noted among individual Roma populations in Slovakia - 1 patient per 40 births, the lowest in Japan, less than 1 case per 100,000 births. The disease is associated with mutations in the PAH gene coding for phenylalanine hydroxylase. The gene is localized on the long arm of the chromosome 12. To date, more than 950 mutations have been described in the phenylalanine hydroxylase gene, the frequency and occurrence of which is characterized by significant interpopulation differences, but only a few of them occur at a frequency of more than 1%. Among Europeans, the most common is missense mutation in the 12th exon of the PAH gene R408W. The type of inheritance of phenylketonuria is autosomal recessive, that is, there is a 25% risk of the birth of second child with PKU. Since the frequency of carriage of a relatively small number of major mutations in the PAH gene is high, in some countries screening for the most frequent mutations is suggested to be performed for all couples planning to have children.

Phenylketonuria diagnostic

The screening allows timely detection of the disease, to begin treatment and thereby prevent pathological changes in the central nervous system in children with PKU. The blood for screening is taken from the newborn's heel, and the level of phenylalanine (PA) is determined in the laboratory. If the level of PA is high, additional studies are performed to confirm the PKU. The last step in refining classical phenylketonuria is molecular genetic diagnosis. In this regard, laboratories which perform neonatal screening for PKU need kits to detect frequent mutations in the PAH gene.

Indications for genetic analysis:

- in a complex of diagnostic methods for the formulation or verification of the **Phenylketonuria** diagnosis;
- for preimplantation and prenatal diagnostics of **Phenylketonuria** with known mutations in the proband, parents of the proband;
- definition of carriers of mutations among relatives of patients with **Phenylketonuria** in case planning pregnancy;
- as part of a complex of measures during pregnancy planning, especially in cases of marriage between relatives.

It should be borne in mind that not finding mutations does not exclude a diagnosis **Phenylketonuria**, because the patient may have extremely rare mutations that can not be identified using this panel.

«DNA-Technology» has developed Phenylketonuria Screen REAL-TIME PCR Genotyping Kit for detection of the 4 most common genetic polymorphisms of PAH gene associated with inherited risk of phenylketonuria «Phenylketonuria Screen» (see Table 35 and 36).

Table 35. Phenylketonuria Screen REAL-TIME PCR Genotyping Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Phenylketonuria Screen	–	*	*	–	RUO

*** Note:**

RUO (research use only)

Table 36. Technical characteristics and content of the kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT-polymerase	1 tube (96 mcl)
PCR buffer	2 tube (960 mcl)
Mineral oil	1 vial (3,84 ml)
Polymotphisms to be identified	1 tube PAH: R261Q – 960 mcl 1 tube PAH: R408W – 960 mcl 1 tube PAH: IVS10nt546 – 960 mcl 1 tube PAH: IVS12+1G>A – 960 mcl
Specimen for analysis	Whole blood
Shelf life	12 months
Storage temperature	+ 2…+ 8 °C – 20 °C (for Taq-AT-polymerase)

Technology:
- PCR melting;
- Use of other technological platforms is not permitted.

DNA extraction kits:
- *PREP-GS GENETICS*.

Minimum amount of DNA for analysis:
- 1.0 ng for amplification tube.

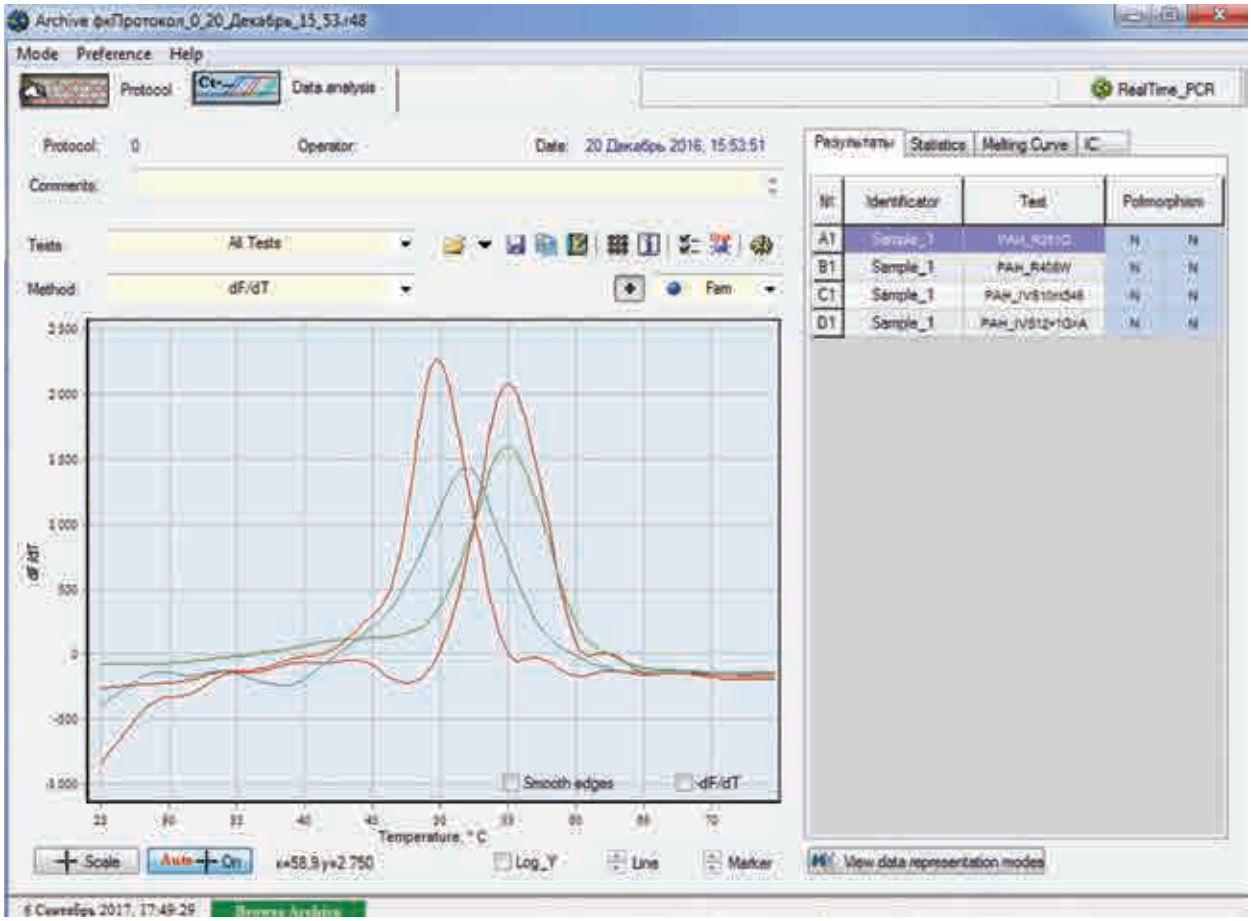
The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Advantages of the Phenylketonuria Screen REAL-TIME PCR Genotyping Kit for detection of the 4 most common genetic polymorphisms of PAH gene associated with inherited risk of phenylketonuria («Phenylketonuria Screen»):
- processability (standard PCR methodology with the detection of results in real time);;
- high speed (it takes less than day to determine the patient's genotype);
- automatic output of results (for DT devices);
- low cost of analysis;
- high sensitivity (technology allows to reliably distinguish the allelic variants of the gene from each other);
- simultaneous detection– in one tube two allelic variants of the gene in one tube;
- internal control (IC) allows to estimate the amount of human genomic DNA in the amplification tube and avoid mistakes during genotyping.

Equipment required for analysis:
The kit is intended for use in the laboratories **equipped with detecting Real-time thermo cyclers (DT devices produced by « DNA-Technology »)**: DTlite, DTprime

Devices of **DT series** are equiped with special English software, which supports **automatic** data processing and delivery of research results in an easy to interpret format (Fig. 21).

A



In addition, the program allows to issue results in a convenient and understandable form for data analysis by clinicians.

B

№	Name of research	Results
		Genotype
1	PAH_R261Q	N N
2	PAH_R408W	N N
3	PAH_IVS10nt546	N N
4	PAH_IVS12+1G>A	N N

Fig. 21. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.3. Reproductive genetics

According to WHO, about 15 % of married couples seek medical help for infertility every year.

Infertility is the inability of a man or woman of childbearing age to reproduce an offspring. A couple is considered infertile if the woman cannot get pregnant within one year of regular unprotected sex (at least once per week). Infertility is classified into:

- Primary infertility: the couple never became pregnant;
- Secondary infertility: the couple became pregnant ending with childbirth, miscarriage, stillbirth, etc;
- Absolute infertility;
- Relative infertility.

On average, about 5 % of couples experience absolute infertility, when treatment does not achieve results [125].

Infertile marriages are often characterized by genetic disorders that can be caused by endocrine disorders, anatomic genital abnormalities and spermatogenesis disorders. Fast and accurate diagnosis is important in choosing effective treatment.

It is important to remember that overcoming infertility amid genetic disorders can lead to birth of a sick child. Correct diagnosis would help conceive and give birth to a healthy baby. Gynecologists and andrologists must have sufficient knowledge of the genetic causes of infertility so that they will be able to give appropriate recommendations to couples wishing to have children [148].

12.3.1. AZF Microdeletions Real-Time PCR Genotyping Kit

Male infertility is inability of a man to impregnate a woman.

Genetic disorders are present in 30-50% of cases where oligozoospermia, azoospermia and other severe disorders are detected via semen analysis (baseline study in a barren marriage) [125].

Y-chromosome deletion in the AZF (azoospermia factor) region is the most common genetic factor of male infertility.

In men with azoospermia or oligozoospermia, microdeletions can be present in the three loci of the Y-chromosome – **AZFa**, **AZFb** and **AZFc** (Fig. 22). These deletions are extremely rare for normospermia and for sperm concentration > 5 million/ml.

Y-chromosome microdeletions are not detected via cytogenetic analysis, thus making their molecular genetic search reasonable. The European Academy of Andrology (EAA) recommends testing all men with azoospermia and severe oligozoospermia (< 5 million sperm per milliliter of semen) for the presence of AZF deletion [72].

The **AZFa subregion** contains three genes named UTY, USP9Y and DBY, whose deletions lead to azoospermia with Sertoli cell-only syndrome type 1 (absence of germ cells in the seminiferous tubules), which is typical for complete AZFa deletion [47, 121].

It should be noted that AZFa does not contain repetitive sequences and its deletions occur with low frequency (about 5 % of all Y chromosome microdeletions). STS markers sY84, sY86 and sY615 are sufficient to identify AZFa deletions. The use of at least two markers – sY84 and sY86 – is diagnostically significant [47, 121, 122].

Procedures for surgically isolating spermatozoa are ineffective in men carrying these deletions.

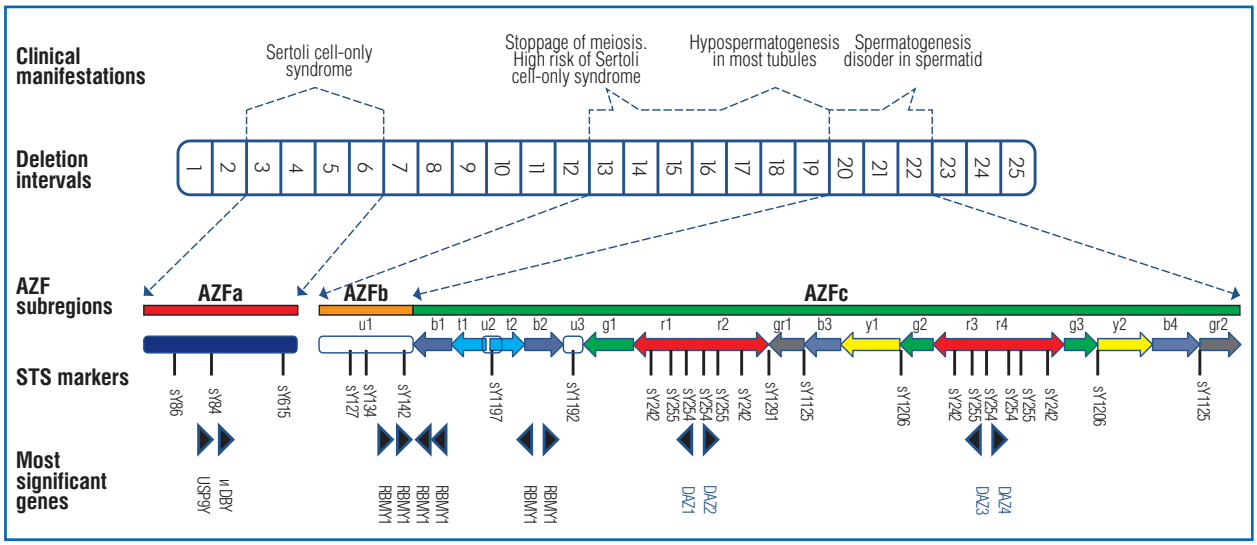


Fig. 22. Schematic representation of the AZF region

The **AZFb subregion** contains sequences that are represented both with a single copy and in the form of high-repeating direct and inverted palindromic sequences. AZFb deletions occur in about 16 % of all Y chromosome microdeletions.

The multi-copy RBMY gene (30 to 40 copies), whose deletions are detected in men with azoospermia or severe oligozoospermia, is mapped in this subregion. The STS markers in this subregion are sY127 and sY134. In line with the guidelines of the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN), loss of these markers goes with severe spermatogenesis disorders with high risk of the Sertoli cell-only syndrome type I [21].

Partial deletions of the proximal portion of the AZF region are characterized by more severe clinical symptoms than distal deletions. Complete AZFb deletion, which leads to delay in sperm maturation during spermatogenesis is of major clinical significance. In this case, TESA forecast is unfavorable.

AZFc deletions are the most common (over 60 % of all Y chromosome microdeletions). AZFc consists of blocks of high-repetitive sequences, which are organized into palindromic structures, with total length of $3.5x10^6$ bps. [99].

One of the key genes in this subregion is the DAZ gene. The b2/b4 deletion leads to loss of all its copies. Markers sY254, sY255, sY1291, sY1206, sY1197 and sY1125 are used to identify it.

The histologic picture of the testicle in b2/b4 deletion may be different as spermatogenesis is blocked much less frequently than in AZFa and AZFb deletions. Sertoli cell-only syndrome type II (minimal number of spermatogenic cells in the seminiferous tubules) is rarely detected. Therefore, spermatozoa can be detected both in the testicle and in the semen [84, 121].

One more Y deletion type – **gr/gr (sY1291) deletion** – is described in the AZFc locus. In this case, half of the AZFc subregion drops out, which changes the number of copies of genes located inside this region. The risk of oligozoospermia is seven times higher in carriers of gr/gr deletions. Testicular germ cell tumors may develop [33, 98, 106].

Microdeletions of several subregions of the Y chromosome are found in 15% of cases. They almost always lead to azoospermia and Sertoli cell-only syndrome. Moreover, spermatogenesis disorders in the case of AZFb and AZFc distal deletion may be less severe [47].

ATTENTION!

Among identified markers, there are some groups associated with rigid adhesion:

- sY84, sY86,
- sY127, sY134,
- sY254 and sY255

Deletion of only one marker from the group almost does not occur. Each case, where deletion of only one of the two markers is established, must be primarily regarded as PCR artifact and requires another study (Fig. 23) [8].

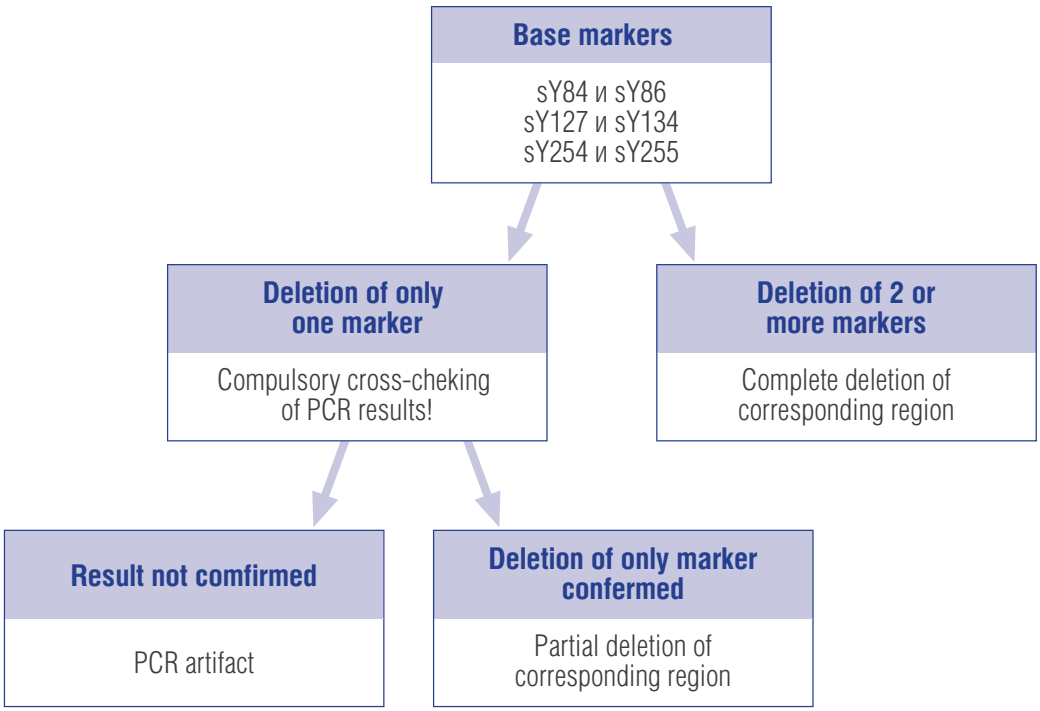


Fig. 23. Block diagram for lab self-control, with indication of analytical steps (Krausz C., 2014)

In identifying the genetic causes of infertility, particularly partial AZF deletions, infertility can be overcome by intracytoplasmic sperm injection (ICSI). It should be noted that in the case of conception, Y chromosome deletion will surely be passed to all the sons of the man, and the size of their microdeletions may be more extensive up to complete deletion. In this regard, it is necessary to observe boys born via ICSI to assess their fertile status.

If AZF microdeletions are detected in the father through assisted reproductive technology, preimplantation genetic diagnosis and transfer of female embryo are recommended [128].

Indications for genetic analysis:

- Examination of infertile couple in the complex of diagnostic methods;
- Selection of adequate methods of overcoming infertility;
- Assessment of the probability of sperm release during TESE, MESA, PESA or TESA;
- Assessment of the risk of fertility disorders in sons.

DNA-Technology developed a kit (see Tables 37-39) for detecting AZF deletions associated with male infertility. The analytical panel included 13 nonpolymorphic markers that allow detecting deletions in all AZF loci.

Table 37. AZF Microdeletions Real-Time PCR Genotyping Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
AZF Microdeletions	–	–	*	–	CE/IVD

*** Note:**

RU/IVD – kits for *In Vitro* Diagnostic, which are registered in Russia only

CE/IVD – kits for *In Vitro* Diagnostic, which are registered in EU

RUO – kits for Research Use Only

Table 38. Strip content, colour codes and detection channels

№ of tube in strip	Dye label/ detection channel					Colour of the PCR-mix	Paraffin color
	Fam	Hex	Rox	Cy5	Cy5.5		
1	sY134	sY242	–	–	–	Blue	White
2	sY142	sY255	–	–	–	Colorless	
3	sY615	sY254	–	–	–		
4	sY1125	sY84	–	–	–		
5	sY1197	sY86	Marker	–	–		
6	sY1206	sY127	–	–	–		
7	sY1291	–	–	–	–		
8	SRY	SIC	–	–	–		

Table 39. Supply complete set of AZF Microdeletions Real-Time PCR Genotyping Kit

Number of tests	24 tests
Kit format	Aliquoted
Taq-polymerase	4 tubes (480 mcl each)
PCR buffer	A mixture for amplification – 24 strips of 8 tubes each (20 mcl each)
Mineral oil	4 tubes (960 mcl each)
Types of genotyping covered	1 tube – loci sY134, sY242 2 tubes – loci sY142, sY255 3 tubes – loci sY615, sY254 4 tubes – loci sY1125, sY84 5 tubes – loci sY1197, sY86, Marker 6 tubes – loci sY1206, sY127 7 tubes – loci sY1291 8 tubes – SRY, SIC
Specimen for analysis	Whole blood

Technology:
Real-time polymerase chain reaction.

Equipment required for analysis: DT devices produced by DNA-Technology: DTlite, DTprime

Additional reagents:

- Positive sample control (K+) – 1 tube (150 mcl) – is included in the kit;

Shelf life: 12 months.

Storage temperature: +2 to +8 °C.

DNA extraction kits:

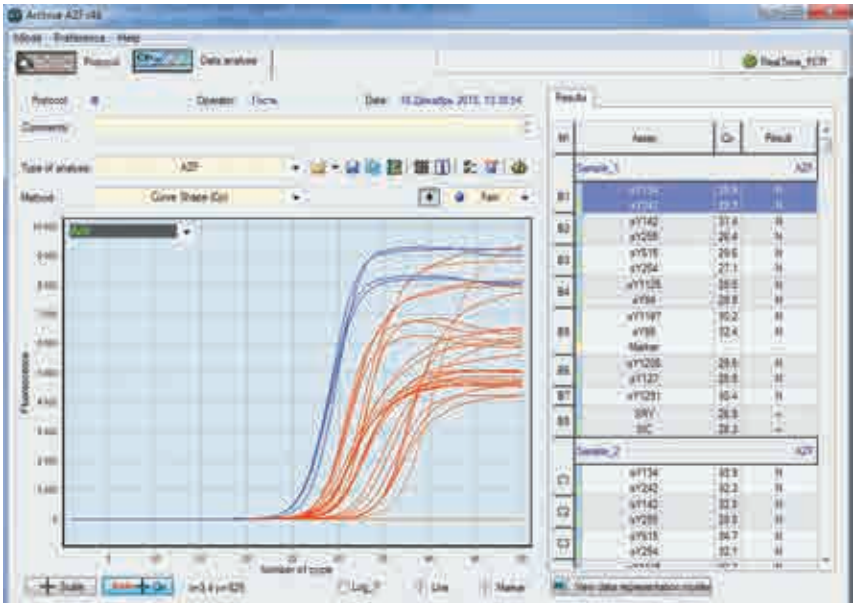
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig.24).

A



B

№	Name of marker	Loci	Result
1	sY86	AZFa	Norm
2	sY84	AZFa	Norm
3	sY615	AZFa	Norm
4	sY127	AZFb	Norm
5	sY134	AZFb	Norm
6	sY142	AZFb	Norm
7	sY1197	AZFc	Norm
8	sY254	AZFc	Norm
9	sY255	AZFc	Norm
10	sY1291	AZFc	Deletion
11	sY1125	AZFc	Norm
12	sY1206	AZFc	Norm
13	sY242	AZFc	Norm

Fig. 24. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4. Genetics of multifactorial disorders

Multifactorial disorders or diseases with hereditary predisposition are caused by interaction of certain combinations of alleles of different loci and specific environmental factors. Multifactorial diseases include hypertension, stroke, thrombosis, most cardiovascular diseases and tumors. Pregnancy complications are also multifactorial in nature [14].

Carriage of risk alleles and presence of precipitating factors play an important role in multifactorial diseases. Not all carriers of risk alleles become ill, but those whose factors harmful to that individual are superimposed on the predisposing genetic background. The trigger can be pregnancy, eating habits, medications, lifestyle, and other factors.

Note that detection of genetic characteristics of a person (genetic risk factors) does not mean that the disease is present or absent.

Personal results of genetic study of a patient should be transferred to him only after preliminary clarifications and consultation with a doctor. Assessment of the genetic characteristics of a particular patient is the exclusive responsibility of the doctor. Such assessment can be based on the overall knowledge about the state of the patient's health and lifestyle.

Detection of genetic polymorphisms and individual genetic passport preparation allows implementing the concept of **personalized medicine**, giving **personalized** recommendations for prevention of the disease, assessing the need for closer medical supervision, and assigning additional examination and consultation by experts.

In some cases, a timely change in lifestyle, diet or use of pharmacological agents can prevent or significantly reduce the severity of multifactorial diseases. It is important to remember that only a doctor can decide whether more screening and/or expert consultation is needed, the doctor also decides whether the patient needs to change his/her lifestyle and treatment.

Considering the fact that several genes usually contribute to development of multifactorial diseases, it is necessary to investigate the collection of genetic polymorphisms, integrated into the gene network.

A **gene network** is a group of genes working in the coordinated manner, ensuring formation of phenotypic characteristics of the organism. For example, it is known that ischemic heart disease can occur through several ways (Fig. 25).

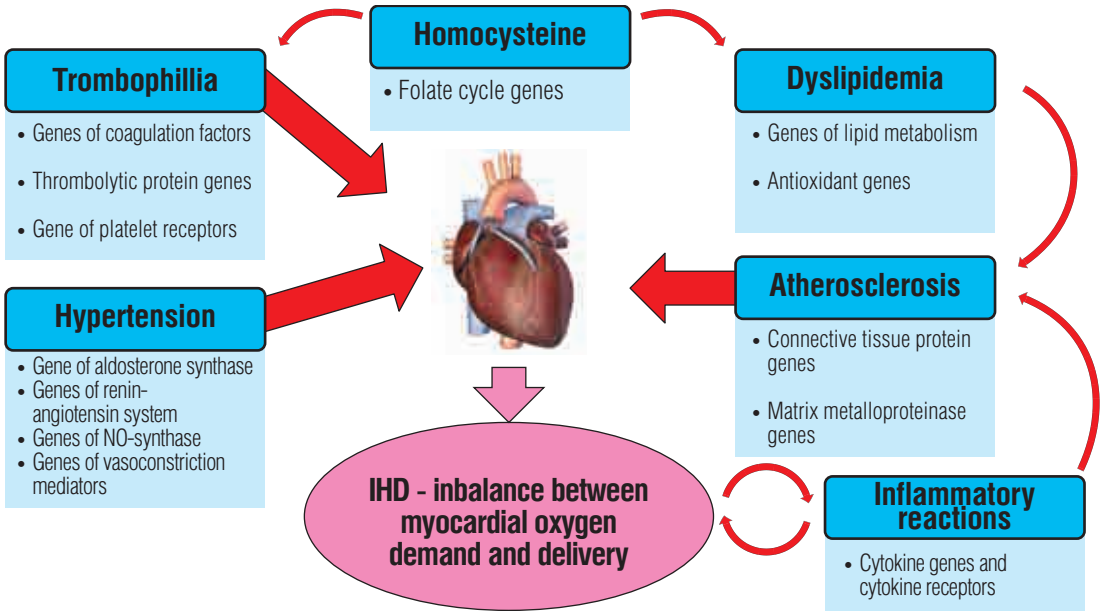


Fig. 25. Gene network. Cardiogenetics. Ischemic heart disease

The main links of pathogenesis are tendency to hypertension, dyslipidemia, and subsequent atherosclerosis, and thrombophilia. In recent years, much attention has been paid to involvement of individual characteristics of the immune system and features of folate metabolism in IHD. The list of the needed genetic markers for analysis is determined depending on the purpose.

For example, in the case of analysis of hypertension as an independent nosology (Fig.26), the pathogenesis can be investigated in more detail, and this would require in-depth genetic study.

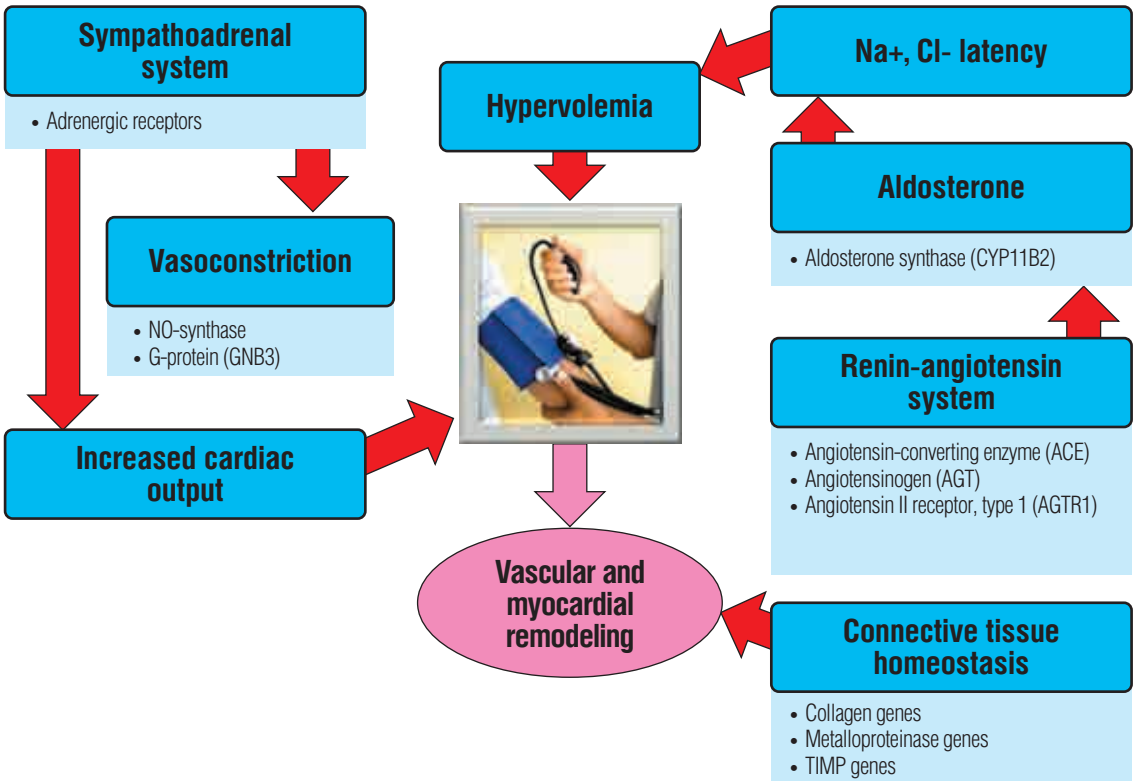


Fig. 26. Gene network. Cardiogenetics. Hypertention

Thus, when studying a multifactorial pathology, formation of ***screening package*** (see Table 40) serves as the ground for clinically reasonable genetic test. This format allows the clinician to quickly obtain detailed information on the most likely etiology and pathogenesis and its leading link for a particular patient. This can help determine individual tactics and allow to choose the most effective treatment for a patient.

Table 40. Principle of formation of a screening package

Indications \ Recommended set	Thrombophilia Susceptibility	Hypertension Susceptibility	Folate Metabolism	Lactose Intolerance	Warfarin Pharma-cogenetics	Clopidogrel Pharma-cogenetics	BRCA	CHEK2	IL28B	Osteo-porosis
Obstetrics and gynecology										
Pregnancy problems and a history of fetal anomalies or their presence in relatives	✓	✓	✓							
Pregnancy planning			✓							
Prescription of oral contraceptives and HRT	✓									
Indications for IVF procedures	✓		✓							
Postmenopausal osteoporosis	✓			✓						✓
Cardiology										
IHD	✓	✓	✓							
Cerebrovascular disorders	✓	✓	✓							
Hypertension		✓	✓							
Cases of history of massive bleeding or its presence in relatives	✓									
History of thrombosis and throm-boembolism or their presence in relatives	✓		✓							
Start of clopidogrel therapy						✓				
Start of warfarin therapy					✓					

Indications \ Recommended set	Thrombophilia Susceptibility	Hypertension Susceptibility	Folate Metabolism	Lactose Intolerance	Warfarin Pharma-cogenetics	Clopidogrel Pharma-cogenetics	BRCA	CHEK2	IL28B	Osteo-porosis
Surgery										
Major surgery, prosthetic repair of joints	✓									
Oncology										
Breast and ovarian cancers	✓	✓	✓				✓	✓		
Prescription of chemotherapy for various tumors	✓		✓							
Pediatrics / Neonatology										
Lactose intolerance				✓						✓
Neonatal ischemic stroke	✓									
Prolonged bleeding from umbilical wound, bleeding diathesis	✓									
Spina bifida, facial skeleton diseases	✓									
Infectious diseases										
Treatment of chronic hepatitis C virus genotype 1									✓	
Endocrinology										
Lactose intolerance				✓						✓
Postmenopausal osteoporosis				✓						✓
Fragility fractures at the age of 50 years and above				✓						✓

The advantage of the *screening package* is saving costs due to the use of ready-to-use examination technology: cost of a single test, included in the set, is significantly lower the cost of single test, which is used separately. From a technological point of view, this format is effective when conducting screening and multiparameter studies. It is ergonomic when working with large flow.

Additional laboratory tests are also recommended to evaluate the current condition of a patient.

Indications for genetic test:

- Belonging to the risk group;
- Familial nature of the disease;
- Atypical / early onset of the disease;
- Tolerance to therapy.

Advantages of detecting genetic polymorphisms via real-time PCR:

- Efficient (standard real-time PCR techniques);
- High speed;
- Automatic delivery of results (*for DT devices*);
- Low cost of analysis;
- High sensitivity;
- Simultaneous detection – two allelic variants of one gene are detected in one tube;
- *C (intake control)* – allows you to estimate the amount of DNA in amplification tube and eliminate genotyping errors.

DNA-Technology offers the following kits (see Table 41) for detecting genetic polymorphisms using real-time PCR.

Table 41. Kits produced by DNA-Technology for detecting genetic polymorphisms using real-time PCR

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Hypertension Susceptibility	–	–	*	–	CE/IVD
Thrombophilia Susceptibility	–	–	*	–	CE/IVD
ThrombophiliaSusceptibility F2, F5 mutations	–	–	*	–	CE/IVD
Folate Metabolism	–	–	*	–	CE/IVD
Lactose Intolerance	–	–	*	–	CE/IVD
Calcium Metabolism	–	–	*	–	RU/IVD
Warfarin Pharmacogenetics	–	–	*	–	CE/IVD
Clopidogrel Pharmacogenetics	–	–	*	–	RUO
BRCA SNP genotyping	–	–	*	–	CE/IVD
CHEK2 SNP genotyping	–	–	*	–	RUO
Immunogenetics. IL 28B	–	–	*	–	CE/IVD
Osteoporosis	–	–	*	–	RUO

*** Note:**

RU/IVD – kits for *In Vitro* Diagnostic, which are registered in Russia only

CE/IVD – kits for *In Vitro* Diagnostic, which are registered in EU

RUO – kits for Research Use Only

Technology: melting.

Kit format: Rt – not pre-aliquoted.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

Shelf life:
Rt – 6 months (for a kit of CHEK2 control samples – 12 months from date of manufacture)

DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Specimen for screening: Whole peripheral blood.

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

Additional reagents:
- positive test samples.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time
- Strip plastic rack and centrifuge (vortex) rotor.

Equipment required for analysis:
DT devices produced by DNA-Technology DTlite, DTprime, DT-96.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology).

12.4.1. Oncogenetics

Cancer is a major social and health problem because of high incidence and mortality. Moreover, it is obvious that the effectiveness of cancer treatment is higher in the early stages of the disease. Therefore, timely diagnosis is an urgent task and it helps to significantly reduce mortality rates.

It is known that oncological diseases are hereditary in significant percentage of cases and they are associated with carriage of mutations in certain genes received from one parent. Carriage of oncogenic mutations is widespread and they are found in 1-2 % of people in all populations.

Hereditary mutations are characterized by the frequency of occurrence in the population as a whole, and also by the degree of their penetrance (see Table 42). Penetrance reflects the probability that the carrier of that genetic marker will develop the disease, in this case cancer. The stronger the penetrance, the higher the probability is.

- **Class I** (High-penetrance) mutations are rare in the general population, but have high penetrance. Example: BRCA1 and BRCA2 gene mutations in patients with breast cancer and/or ovarian cancer. About 50-70 % of inherited cases of such cancer are caused by mutations in one of these genes (often BRCA1) [46].
- The **second class** (Moderate-penetrance) of inherited oncogenic mutations has an average risk of disease. These mutations are also quite rare in the general population.
- The **third class** (Low-penetrance) is widespread in the population. The clinical significance of identifying this class of mutations is largely determined by the presence of additional risk factors.

Table 42. Genetic predisposition to breast cancer [10; 16; 17].

Penetrance	Risk increase	Genes
High-penetrance	5–20 times	BRCA1, BRCA2
Moderate-penetrance	1.5–5 times	CHEK2, ATM, PALB2, NBN, RECQL3
Low-penetrance	Up to 1.5 times	ESR1, FGFR2, TOX3, LSP1, MAP3K1

12.4.1.1. BRCA SNP genotyping Kit

There are over 1 million annual cases of breast cancer in the world. In Russia, 50 thousand cases are recorded annually. Breast cancer incidence in Russia is 42.7 per 100,000 population (standardized figures for 2007) [7].

High mortality from breast cancer is one of the most common problems. There is almost 13% mortality in the first year of diagnosis. Obviously, efficiency of treatment is higher in the early stages of the disease. Timely diagnosis is therefore an urgent task and could reduce deaths significantly [7].

Note that breast cancer is an extremely heterogeneous pathology (Fig. 27).

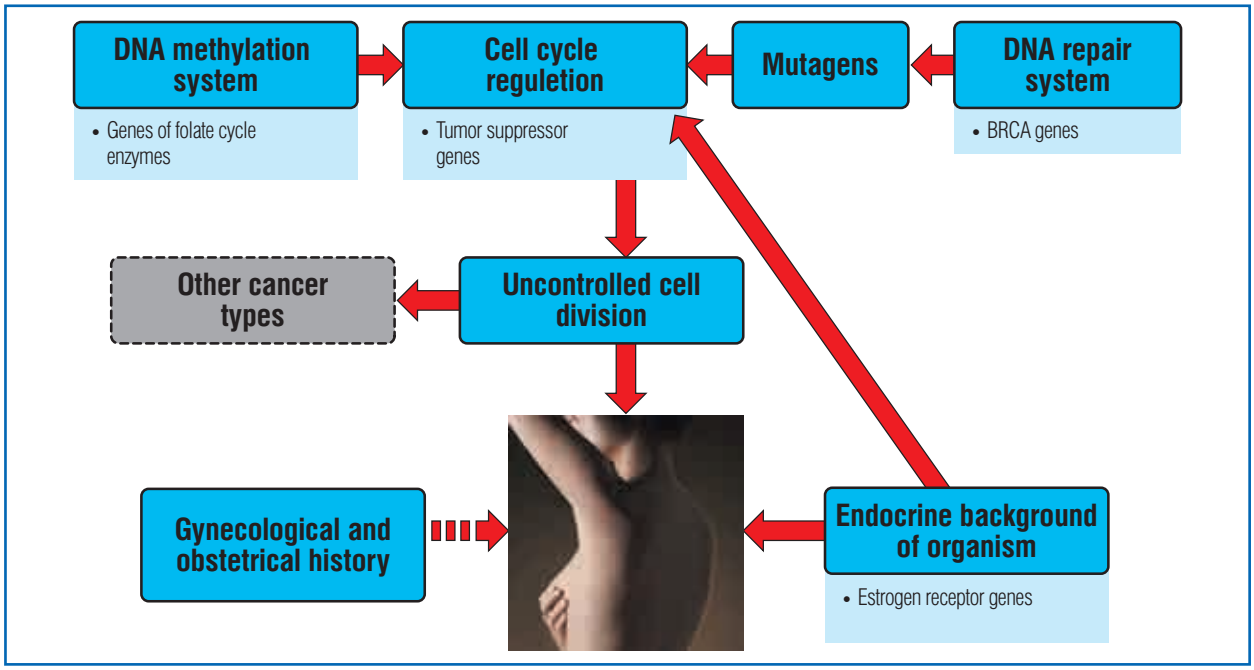


Fig. 27. Gene network. Oncogenetics

It is known that 5-10 % of cases of breast cancer and ovarian cancer are hereditary and can be attributed to **BRCA1** and **BRCA2** gene mutations. Both genes increase the risk of breast cancer by 80-85% in women as they approach 80 years of age [9].

BRCA1 and BRCA2 genes encode ubiquitously expressed nuclear proteins. It is estimated that the function of these proteins involves regulation of DNA repair and maintenance of genome integrity. Families carrying BRCA1 and BRCA2 mutations show autosomal dominant inheritance of tumors [79].

It was shown that BRCA1 – associated with breast cancer – unlike sporadic cancer, has a higher degree of malignancy, a high incidence of estrogen and progesterone-negative tumors, incidence of medullary cancer pronounced in the form of lymphoid infiltration, pronounced therapeutic pathomorphism up to complete regression. It is established that the survival rate of patients with hereditary cancer of the female reproductive system is significantly higher than in the total group of patients, regardless of the stage of treatment: 5-year survival rate of patients with hereditary breast cancer is 75 % (and 43 % for all other forms of cancer) [8].

BRCA1 and BRCA2 genes are not strictly specific for breast cancer. Pathological genotype BRCA1/2 increases the risk of developing ovarian cancer, stomach cancer, colon cancer, pancreatic cancer, bladder cancer, head and neck tumors, endometrial cancer, biliary tract cancer, as well as melanoma [140].

Indications for molecular genetic test:

- Breast cancer at a young age (before 50 years);
- Burdened family history (two or more blood relatives with breast and/or ovarian cancer);
- Primary-multiple malignancies in the patient or his relatives:
  - Bilateral breast cancer;
  - Breast cancer and ovarian cancer;
- Other morphological features of breast cancer:
  - Three times negative breast cancer (tumors ER-, PR-, HER2/neu);
  - Medullary carcinoma;
  - Ovarian cancer;
  - Breast cancer in men in personal and family history.

DNA-Technology, together with the Blokhin Russian Cancer Research Centre, conducted joint research to determine the incidence of 11 previously described BRCA1 and BRCA2 gene mutations in an unselected sample of breast cancer patients in the Russian population on sampling of 1091 people [1].

Based on the results of this study, a kit was developed for determining polymorphisms associated with the risk of developing breast and ovarian cancer by real-time PCR method (see Tables 43 and 44).

Table 43. Genetic polymorphisms associated with breast cancer

Gene	Polymorphism (mutation)	Risk allele	Incidence		Risk assessment for different genotypes
BRCA1	185delAG	delAG	0,1%	Overall incidence of about 5.9% in an unselected sample (1091 people)	Ins/Ins – population risk Ins/Del – high risk
	4153delA	delA	0,7%		Ins/Ins – population risk Ins/Del – high risk
	5382insC	insC	4,0%		Del/Del – population risk Del/Ins – high risk
	3819delGTAAA	delGTAAA	0,2%		Ins/Ins – population risk Ins/Del – high risk
	3875delGTCT	delGTCT	0,1%		Ins/Ins – population risk Ins/Del – high risk
	300T>G (Cys61Gly)	G	0,4:%		TT – population risk TG – high risk
	2080delA	delA	0,2%		Ins/Ins – population risk Ins/Del – high risk
BRCA2	6174delT	delT	0,2%		Ins/Ins – population risk Ins/Del – high risk

Table 44. Specifications and supply complete set of the kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (192 mcl)
Mineral oil	1 vial (7,68 ml)
PCR buffer	1 vial (3,84 ml)
Polymorphisms to be identified	BRCA1: 185delAG – 960 mcl BRCA1: 4153delA – 960 mcl BRCA1: 5382insC – 960 mcl BRCA1: 3819delGTAAA – 960 mcl BRCA1: 3875delGTCT – 960 mcl BRCA1: 300T>G – 960 mcl BRCA1: 2080delA – 960 mcl BRCA2: 6174delT – 960 mcl
C+1 (homozygous for normal allele) C+2 (heterozygous)	1 tube (270 mcl each) 1 tube (270 mcl each)
Specimen for analysis	Whole blood

Technology:
- PCR melting;
- Use of other technological platforms is not permitted.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, DT-prime, DT-96.

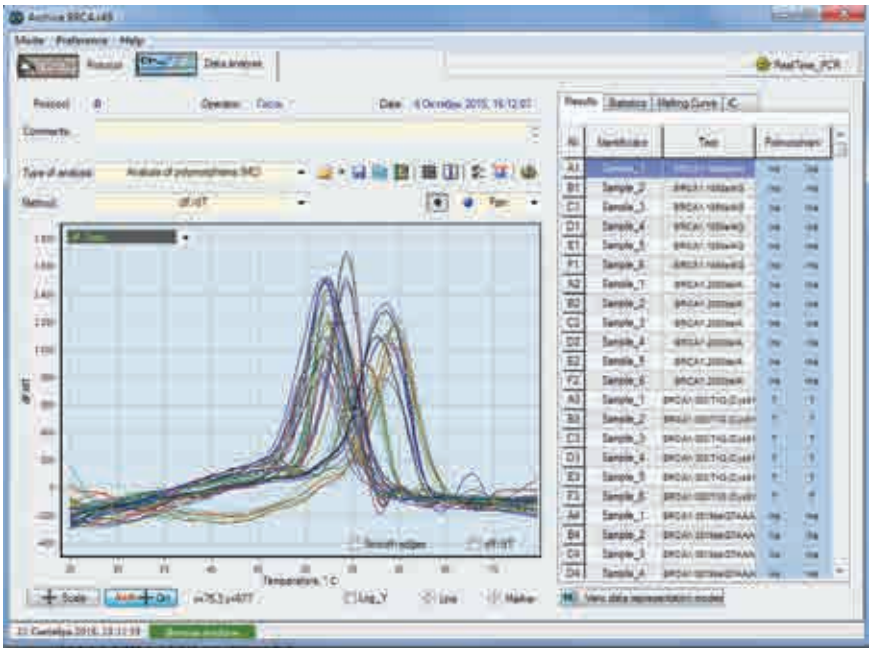
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 28).

A



B

Evaluation of genetic predisposition to BRCA-associated breast and ovarion cancer

Date 6 Октября 2015, 16:12:07
Nomber of tube
Patient name
Sex
Age
Organization
Clinician name
Comments

logotype

information about laboratory

Sample ID: Sample_1

№	Name of research	Genotype	Note
1	BRCA1:185delAG	Ins/Ins	norm
2	BRCA1:2080delA	Ins/Ins	norm
3	BRCA1:300 T>G (Cys61Gly)	T/T	norm
4	BRCA1:3819delGTAAA	Ins/Ins	norm
5	BRCA1:3875delGTCT	Ins/Ins	norm
6	BRCA1:4153delA	Ins/Ins	norm
7	BRCA1:5382insC	Del/Del	norm
8	BRCA2:6174delT	Ins/Ins	norm

Conclusion:

Mutation in gene BRCA1 (185delAG, 2080delA, 300 T>G (Cys61Gly), 3819delGTCT, 3875delGTCT, 4153delA, 5382insC) are not detected.

Fig. 28. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4.1.2. CHEK2 SNP genotyping Kit

CHEK2 gene mutations belong to Class II mutations in genes that are involved in processes of DNA replication, transcription, recombination and reparation. The genes encode various enzymes (e.g. polymerases, helicase, topoisomerase, etc.) and cell cycle regulating proteins. Defects in these genes are result in chromosomal instability and therefore can lead to different cancer diseases [53].

The clinical significance of detection of CHEK2 gene mutation is not lower than in BRCA1 and BRCA2 genes, especially in the case of familial cancers. It is recommended to carry out second-line test for a number of mutations in these genes if a negative result for carriage of BRCA1 and BRCA2 mutations is obtained. Furthermore, a number of genetic syndromes, whose main characteristic is a predisposition to chromosomal instability, are described. For some of such syndromes, association with heterozygous carriage of CHEK2 gene mutation has been proven [30,104,123].

CHEK2 mutations

The CHEK2 gene (cell-cycle checkpoint kinases 2) encodes a protein called cell cycle checkpoint regulator, which is involved in DNA repair processes and in cell division regulating processes. The product of this gene is an enzyme called protein kinase enzyme and is synthesized in response to damage to DNA molecules. The mechanism of action is to block the cell cycle in the G1 phase or launch apoptosis process, while suppressing malignant transformation of cells. CHEK2 gene mutations lead to the in expression of defective truncated protein and they are associated with emergence of different inherited forms of malignancies. Three mutations are the most important: In the CHEK2 gene 1100delC, IVS2 + 1G> A and 470T> C Ile157Thr.

1100delC mutation in the CHEK2 gene is prevalent in many countries. Mutant allele frequency is 1.1-1.4 % in the European population. It was shown that 1100delC mutation is associated with breast cancer. A large-scale study by the International consortium *Breast Cancer Case-Control Consortium*, which included over 10,000 breast cancer patients and 9,000 healthy women from five countries, found that the hazard ratio for carriers of 1100delC mutations in the CHEK2 gene is 2.34 [32, 41].

Apart from clear association with breast cancer, it was established that 1100delC mutation is associated with prostate cancer [37].

The **IVS2+1G>A mutation** in the CHEK2 gene is rarer than 1100delC and leads to formation of a non-functional protein. The mutation is associated with emergence of oncological diseases of different localization, first and foremost breast cancer. It is more common among residents of Eastern Europe and North America. In a large sample of Eastern European population (about 2000 patients), it was established that IVS2+1G>A allele is clearly associated with prostate cancer (OR=2.0) [37].

Missense mutation 470T>C (Ile157Thr) in the CHEK2 gene is associated with Li–Fraumeni syndrome, breast and prostate cancers, cancer of the colon and rectum, both sporadic and familial. This mutation is more frequent than 1100delC and IVS2+1G>A, and it is found in the population (with a 4-5 % frequency). Amino acid substitution of isoleucine with threonine reduces the functional activity of the protein. Besides, the mutant protein forms dimers with the wild type protein, thereby reducing its activity. On average, the presence of Ile157Thr mutations of the CHEK2 gene increases the risk of breast cancer to a lesser extent than carriage of other CHEK2 mutations [26, 68, 92].

Indications for genetic test:

- Family history (breast cancer, prostate cancer, or colorectal cancer in first-degree relatives);
- One or more relatives with the same type of tumor;
- Atypical proliferative breast diseases;
- Multiple primary tumors in the same organ;
- Multiple primary tumors in different organs;
- Multiple primary tumors in paired organs;
- Multifocality inside one organ;
- Tumor manifestations at an early age;
- Two or more relatives with rare forms of cancer;
- Two or more relatives with tumor related to family cancer;
- Three or more relatives in two generations with tumors of one localization;
- Negative test result for BRCA1 and BRCA2 gene mutations

If mutations are detected at the lab, it is necessary to see an oncologist or visit cancer specialist centers. There, they will determine the tactics for further inspection and, if necessary, assign prophylactic treatment depending on the patient's age, family history and particular clinical situation.

DNA-Technology has developed a kit (see Table 45) for detecting CHEK2 polymorphisms associated with cancer pathology using real-time PCR technique.

Table 45. Specifications and supply complete set of the kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (48 mcl)
Mineral oil	2 tubes (960 mcl)
PCR buffer	2 tubes (480 mcl)
Polymorphisms to be identified	CHEK2:1100delC/IVS2+1G>A– 960 mcl CHEK2:470 T>C (Ile157Thr) – 960 mcl
Positive sample control (C+) [homozygous for normal allele for all polymorphisms]	1 tube (100 mcl)
Specimen for analysis	Whole blood

Technology:
- PCR melting;
- Use of other technological platforms is not permitted.

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, DT-prime.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

Additional reagents:
- Positive test samples* (see Table 47). The kit consists of four tubes with a screw cap:
  - Test sample № 1 [heterozygous for CHEK2:1100delC] – 1 tube (50 mcl);
  - Test sample № 2 [heterozygous for CHEK2:IVS2+1G>A] – 1 tube (50 mcl);
  - Test sample № 3 [heterozygous for CHEK2:470T>C (Ile157Thr)] – 1 tube (50 mcl);
  - Test sample № 4 [homozygous for mutant allele CHEK2:470T>C (Ile157Thr)] – 1 tube (50 mcl).

Used together with *CHEKS2 mutations kit*

***Table 46. Genotypes, determined in positive control samples**

PCR-mix	Polymorphism (mutation)	Genotype				
		C+	Positive control			
			№ 1	№ 2	№ 3	№ 4
CHEK2:1100delC/IVS2+1G>A	CHEK2:1100delC	Ins/Ins	Ins/Del	Ins/Ins	–	–
	CHEK2:IVS2+1G>A	G/G	G/G	G/A	–	–
CHEK2:470T>C (Ile157Thr)	CHEK2:470T>C (Ile157Thr)	T/T	–	–	T/C	C/C

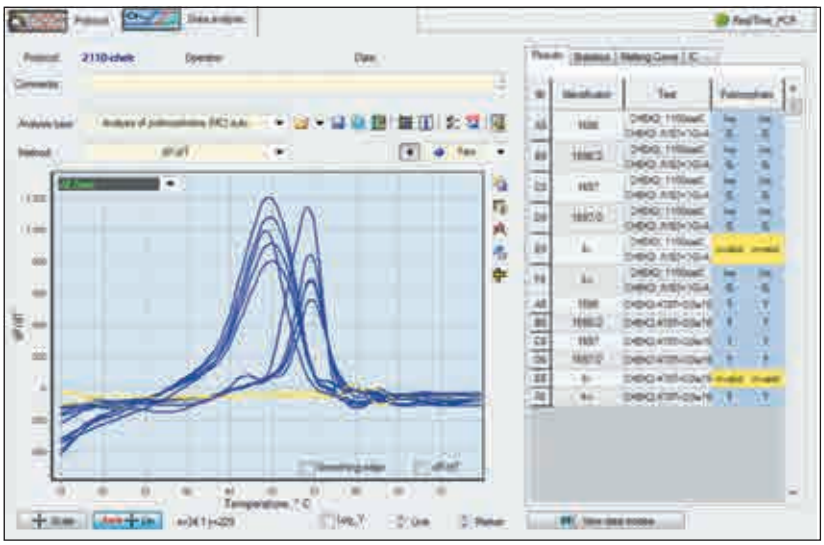
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 29).

A



B

№	Name of research	Genotype	Note
1	CHEK2:1100delC	Ins/Ins	norm
2	CHEK2:IVS2+1G>A	G/G	norm
3	CHEK2:470T>C(Ile157Thr)	T/CI	norm

Fig. 29. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4.2. Hypertension Susceptibility Real-Time PCR Genotyping Kit

Hypertension is a set of conditions that are associated with long increase in hydrostatic pressure in the systemic circulation arteries.

Hypertension is the most common disease among the adult population in developed countries. In Russia, increased blood pressure is diagnosed in 39.2 % of men and 41.1 % of women. 12-15 % of people are found to develop persistent hypertension [15].

There are essential (primary) hypertension or hypertensive disease and symptomatic (secondary) hypertension. Moreover, hypertensive disease accounts for up to 90-95 % of all hypertension cases [12].

Essential hypertension is a multifactorial disease, which is based on genetic polygenic structural defect, causing high activity of long-acting pressor mechanisms [18].

There are three main etiologic risk factors for essential hypertension:

- Adaptation factors;
- External environmental factors;
- *Familial polygenic predisposition* (about 30 % of genetic factors are associated with the functioning of the renin-angiotensin system).

Hypertension is the cornerstone of such serious diseases as myocardial infarction and acute cerebrovascular accident (400,000 cases of stroke are reported in Russia every year). It can also cause permanent lesions of various target organs, leading to chronic diseases of the kidney, eye, heart and brain. The life expectancy of middle-aged patients suffering from hypertension does not exceed 20-30 years, while that of those at high risk is 10 years. Therefore, to improve the quality and length of life, early diagnosis and timely treatment are needed [20].

Having said that, detection of genetic polymorphisms in the genes of the key regulation factors of the cardiovascular system, particularly associated with the functioning of the renin-angiotensin system (RAS), becomes the most urgent task.

Angiotensinogen is an essential component of the renin-angiotensin system, a precursor of angiotensin II – the main effector peptide. Angiotensin-converting enzyme (ACE) hydrolyzes angiotensin I, converting it to angiotensin II. Angiotensinogen and ACE polymorphisms play the key role in the formation of hypertension. Other polymorphic alleles responsible for intracellular transport of ions, regulation of aldosterone synthesis and smooth muscle tone have also been detected.

Indications for genetic analysis:

- Ischemic heart disease (IHD);
- Acute myocardial infarction;
- Stroke;
- Diabetic nephropathy;
- Venous thromboembolism;
- Placental function disorders;
- Microcirculation and vascular tone disorders;
- Diabetes;
- Selection of drugs for hypertension;
- Smoking.

DNA-Technology developed a kit (see Tables 47 and 48) for identification of genetic polymorphisms associated with hypertension using real-time PCR method.

Table 47. Genetic polymorphisms associated with hypertension

Gene	Gene function	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
ADD1 (α-adducin)	A cytoskeletal protein, involved in transporting ions through the cell membrane	**1378 G>T (Gly460Trp)**	**rs4961**	**G/G**	No abnormalities
				G/T	Associated with high sensitivity to changes in sodium balance, relationship with salt-dependent hypertension
				T/T	
AGT (angiotensinogen)	The predecessor of angiotensin II. Has a strong vasoconstrictor effect and increases the total peripheral vascular resistance, thus rapidly increasing blood pressure	**704 T>C (Met235Th)**	**rs699**	**T/T**	No abnormalities
				T/C	Elevated concentration of angiotensinogen to 5 %, which leads to increased level of angiotensin II and hypertension
				C/C	Elevated concentration of angiotensinogen by 11 %, which leads to increased level of angiotensin II and hypertension
		521 C>T (Thr174Me)	**rs4762**	**C/C**	No abnormalities
				C/T	Hypertension
				T/T	
AGTR1 (angiotensin II receptor, type 1)	Angiotensin II receptor, type 1 causes major cardiovascular effects of angiotensin II: vasoconstriction, stimulation of aldosterone synthesis and secretion, sodium reabsorption in the renal tubules, etc.	**1166 A>C**	**rs5186**	**A/A**	No abnormalities
				A/C	Generally, diastolic pressure increases, increased gene expression and increased density of angiotensin II receptors. Homozygotes are found to be more prone to high blood pressure than heterozygotes
				C/C	
AGTR2 (angiotensin II receptor, type 2)	AGTR1 gene is involved in regulation of nitric oxide production. Angiotensin II is the main regulator of aldosterone synthesis	**1675 G>A**	**rs1403543**	**G/G**	No abnormalities
				G/A	Increased sensitivity of angiotensin II receptor, relationship with salt-dependent hypertension
				A/A	

Gene	Gene function	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
CYP11B2 (cytochrome 11b2 – aldosterone synthase)	Aldosterone is involved in regulation of metabolism of sodium and vascular volume and, besides, is a stimulator of cell hypertrophy and fibrosis in the cardiovascular system	**-344 C>T**	**rs1799998**	**C/C**	No abnormalities
				C/T	Polymorphism is located in the promoter region of the gene. Increased gene expression and increased basal production of aldosterone, which increases the risk of salt-dependent hypertension
				T/T	
GNB3 (G-protein beta3 subunit – guanine nucleotidebinding protein beta 3)	Guanine binding protein 3 beta-3 protein or G-protein β-subunit. It plays an important role in intracellular signal transmission	**825 C>T**	**rs5443**	**C/C**	No abnormalities
				C/T	Leads to alternative splicing and synthesis of G-protein truncated at amino acid position 41, resulting in increased proliferative activity and vasoconstriction. Activity of Na-H+-exchanger also increases
				T/T	
NOS3 (nitric oxide synthase)	Nitric oxide synthase of endothelial cells is involved in nitric oxide synthesis by endothelium, thus participating in regulation of vascular tone, blood flow and blood pressure.	**-786 T>C**	**rs2070744**	**T/T**	No abnormalities
				T/C	Reduced activity of the NOS3 gene by 52%. NOS that was formed as a result of this insufficiency is the reason for reduced synthesis and release of nitric oxide, and endothelial dysfunction, which can lead to IHD and acute coronary syndrome
				C/C	
		894 G>T (Glu298Asp)	**rs1799983**	**G/G**	No abnormalities
				G/T	The risk of hypertension at increased plasma pool of the total blood cholesterol is above 209 mg/dl
				T/T	

** Identification in dbSNP National Center for Biotechnological Information, NCBI (USA).*

Table 48. Hypertension Susceptibility Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (216 mcl)
PCR buffer	1 vial (4,32 ml)
Mineral oil	1 vial (8,64 ml)
Polymorphisms to be identified	ADD1: 1378 G>T– 960 mcl AGT: 704 T>C – 960 mcl AGT: 521 C>T – 960 mcl AGTR1: 1166 A>C – 960 mcl AGTR2: 1675 G>A – 960 mcl CYP11B2: -344 C>T – 960 mcl GNB3: 825 C>T– 960 mcl NOS3: -786 T>C – 960 mcl NOS3: 894 G>T – 960 mcl
Specimen for analysis	Whole blood

Technology:
- PCR melting;
- Use of other technological platforms is not permitted.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, DT-prime.

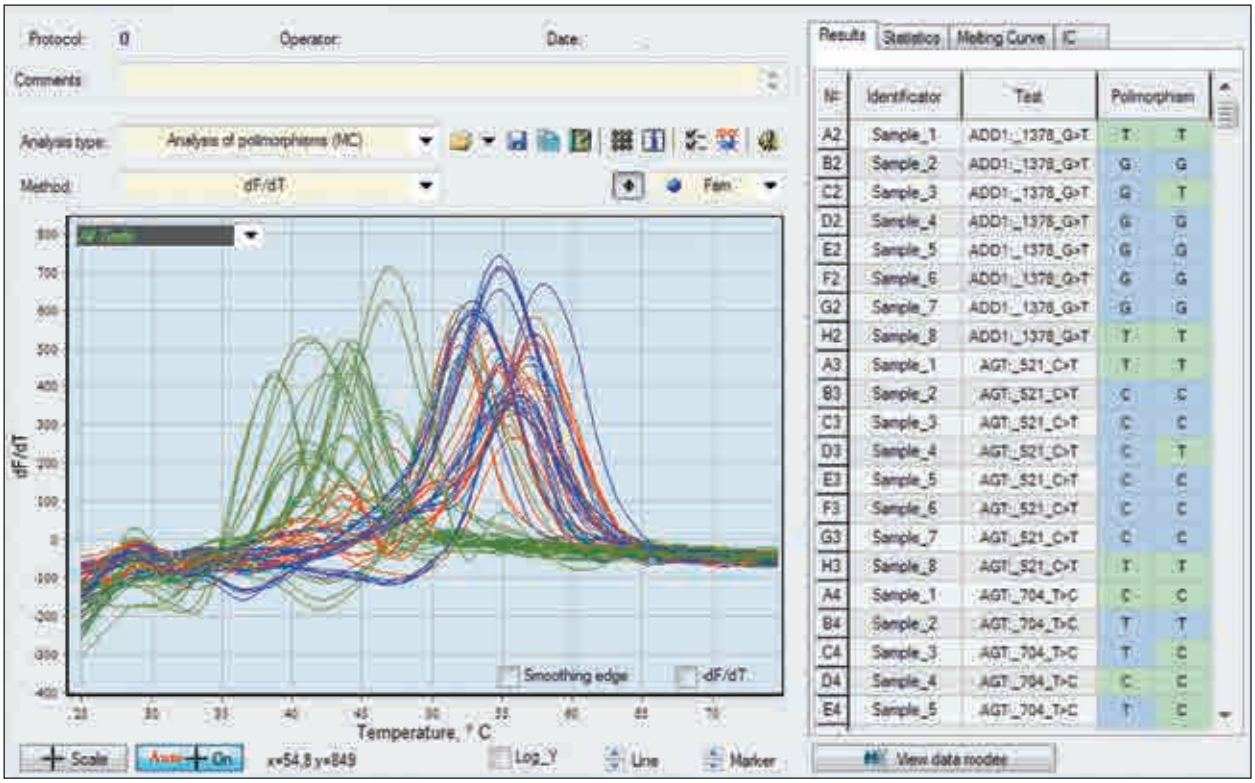
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 30).

A



B

№	Name of research	Results
		Genotype
1	ADD1:_1378_G>T	G G
2	AGT:_521_C>T	C C
3	AGT:_704_T>C	T T
4	AGTR1:_1166_A>C	A A
5	AGTR2:_1675_G>A	G A
6	CYP11B2:_–344_C>T	C T
7	GNB3:_825_C>T	C C
8	NOS3:_–786_T>C	T T
9	NOS:_894_G>T	G G

Fig. 30. Optical measurement analysis results

A – Optical measurement analysis (Fam channel)

B – Analysis report

12.4.3. Thrombophilia Susceptibility Real-Time PCR Genotyping Kit

Thrombophilia (from two Greek words: *thrombus*, meaning "clot", and *philo*, meaning "predisposition") is the state of the blood system, which manifests itself in hemostasis disorder and has a propensity to develop recurrent vascular thrombosis (mostly venous) of different localization and often occurring in association with pregnancy, after surgery, injury or physical overstrain. The disease is caused by genetic (in 30-50 % of thrombotic conditions) or acquired disorders of blood cells, as well as defects in the blood coagulation system.

Genetic predisposition to thrombophilia can occur through genetic defects in both coagulation and anticoagulation (anticoagulant and fibrinolytic) blood systems in which there is a tendency to develop thrombosis.

Thrombosis refers to a lifetime formation of blood clots in the lumen of vessels or in heart cavities.

Thrombosis plays one of the major role in development of cardiovascular diseases, which are first in the list of causes of disability and premature death in economically developed countries. Today, these diseases account for 40-60 % deaths (approximately 14 million deaths each year). Moreover, the ongoing increase in incidence and attack among the young population makes cardiovascular diseases (CVD) the most important medical and social health problem. Mortality from CVD in Russia is 2-4 times higher than in Western European, USA, Canada and Australia. According to statistics in recent years, published on the website http://www.critical.ru, CVDs account for 85.5 % mortality in the Russian population: IHD – 46.8 % and stroke – 38.7 %.

Hereditary thrombophilia plays an important role in the structure of obstetrical and gynecological complications, such as fetal loss, recurrent miscarriage, repeated IVF failure and thromboembolism in pregnant women [55].

Another important issue is prescription oral contraceptives. Oral contraception is one of the most reliable ways to prevent unwanted pregnancy, but carries a risk of thrombosis. It has been shown that hormonal contraception in itself slightly increases the risk of thrombosis, but carriage of a certain genotype increases the danger sharply.

According to the Medical Eligibility Criteria for Contraceptive Use released in 2012 and the fourth edition of the Medical Eligibility Criteria for Contraceptive Use developed by WHO in 2009, to prevent thrombosis and thromboembolic complications in those taking oral contraceptives, it is recommended to detect thrombogenic mutations (F2 – prothrombin mutation, and F5 – Leiden factor) [143].

Genetic analysis allows detecting the gene polymorphisms of factors of the hemostasis system, which lead to abnormal synthesis of the factors or functional activity disorder. This permits to assess the risks of development of cardiovascular disease and obstetrical complications, thromboembolism, venous and arterial thrombosis. Screening of genetic features of thrombophilia helps to early identify patients at risk and make appropriate adjustments in their management tactics.

At the same time, it is important to take into account the population features of the frequency and clinical significance of certain polymorphisms. For example, the prevalence of Factor V Leiden in the European population varies from 2 % to 15 %, with higher rates in Scandinavia and Eastern Mediterranean. The mutation is rare in African, Asian and Indian populations (see Table 49) [28, 73].

Table 49. Prevalence of factor V Leiden mutation in different populations [73]

Population	Prevalence in % (together: heterozygotes + homozygotes)
White European population in general	3-15
Spain	3.3
France	3.8
Germany	4
Iceland	5.2
Great Britain	8.8
Greece	15
Sweden	11
Africa	Absent
Southeast Asia	Absent
Asia Minor	1.2
Indigenous people of Australia	Absent
Japan	Absent
Jordanian Arabs	12.2
Lebanon	14
Western Iran	2.9
Canada	5.3
White population of the USA	5.2
Latin Americans	2.2
Afro-Americans	1.2
Asian population of the USA	0.45
Indigenous people of the USA	1.25

Moreover, besides the F5:1691 G>A variant (Leiden mutation), several clinically significant factor V polymorphisms have been detected in different European and Asian populations (see Table 50).

Table 50. Allelic variants of factor V Leiden [73]

Allelic variant	Nucleotide substitution in factor V DNA	Amino acid substitution in the primary structure of factor V	Clinical significance
R2	4070 A>C	His1299Arg	Increases activated protein C resistance. Associated with the risk of venous thrombosis
Factor V Cambridge	1091 G>C	Arg306 Thr	APC resistance
Factor V Hong Kong	1090 A>C	Arg306Gly	Detected in 4.7 % of Chinese in Hong Kong, with episodes of venous thrombosis in past medical history
Factor V Liverpool	1250 T>C	Ile359Thr	APC resistance

The incidence of prothrombin gene mutation among the European population is between 1 % and 6 %. Prevalence in Southern Europe (3 %) is higher than in Northern Europe (1.7 %). It is rare in people of Asian and African ancestry for whom there is higher association of hereditary thrombophilia with prothrombin S and C deficiency (see Table 51) [28, 73, 112].

Table 51. Population features of association of factors of the hemostasis system with the risk of development of hereditary thrombophilia

Population		F2 G20210A	Antithrombin deficiency (SERPINC1)	Protein S deficiency	Protein C deficiency
Europe Southeast Asia	Healthy	1.7-3 %	0.02-0.15 %	0.03-0.13 %	0.2-0.4 %
	With DVT	6.2 %	1-3 %	1-5 %	3-5 %
Black population in UK	Healthy	0	0.15 %	1-1.2 %	0.13 %
	with DVT	0	5.6 %	18 %	8 %
Europe Southeast Asia	Healthy	0	0	2 %	4 %
	with DVT	0	0.7 %	2.8 %	4.2 %
Black population in UK	Healthy	<0.001 %	–	–	–
	with DVT	1.1 %	–	–	–

Analysis of the contribution of PAI-1 -675 5G>4G gene polymorphism in the development of reproductive problems found that 4G4G allelic variant may be associated with pre-eclampsia (PE), but this dependence has a population feature. According to a meta-analysis, PE risk among African American women is higher than in women of European ancestry, while the Asian population and patients from Spain had a lower risk of PE (see Table 52) [150].

Table 52. Prevalence of allelic variants of the PAI-1 -675 5G>4G gene in patients with pre-eclampsia

Country	Genotypes in PE patients (%)			Genotypes in the control group (%)		
	4G4G	4G5G	5G5G	4G4G	4G5G	5G5G
Brazil	32	34.7	33.3	22.4	44	33.5
Japan	60	32.1	7.8	47.4	40.5	12
South Africa	1.3	27.8	0.7	0.9	22.1	77
Egypt	23.5	69	7.3	1.7	79.7	18.6
Netherlands	33.7	51	15.3	28	55.7	17.2
Italy	38.5	52	9.6	23.7	51.2	25
Germany	32	49	19.1	27.6	63.6	8.7
Finland	25.5	49.6	24.8	29.5	49.5	18
Bulgaria	24	32	44	18.4	63.3	18.4
Scotland	30.5	48.4	21.1	26.2	50.6	23.1

In addition, it was established that the 4G4G variant occurs with high frequency in patients with polycystic ovary syndrome in Turkish, Asian and European populations [75].

It was also found that European patients with pneumonia-induced severe sepsis, the 4G4G allelic variant of the PAI-1 gene is associated with high risk of multiple organ failure and septic shock. The 4G5G genotype is a risk factor for deep vein thrombosis in the European and the Asian and Indian populations.

Investigation of the prevalence of β-fibrinogen gene polymorphism in position -455 (FGB:-455 G>A) in different populations identified a general trend of dominance of GG allelic variants (see Table 53) [39].

Table 53. Frequency of FGB:-455 G>A allelic variants in different populations

Population	Genotype (%)		
	GG	GA	AA
South Asian population in UK (migrants from India, Pakistan, Bangladesh)	66	26	8
White population in UK	56	37	5
Han Chinese	64.6	32.2	3.2
Lebanese	60.6	31.9	7.5
Finns	69.9	24.9	5.2
Koreans	70.4	25.8	3.7
Greeks	69	29	2
Saudi Arabia population	70	25	5

At the same time, there are clear population differences in the significance of allelic variants of the FGB gene polymorphism in terms of elevated plasma fibrinogen concentrations and risk of cardiovascular pathologies and atherosclerosis. It was established that the populations of Saudi Arabia, South Asian and African-Americans have a higher baseline levels of plasma fibrinogen concentrations than white European population and white US population under the presence of the same GG genotype. Moreover, the presence of heterozygous GA variant in South Asian population causes a substantial increase in plasma fibrinogen concentrations, comparable with those of the European population, but with presence of a homozygous form of polymorphism (AA). This dependence is reflected in the clinical manifesta-

tions of cardiovascular diseases, including severity and frequency of deaths [39, 63, 112].

In addition, meta-analysis carried out on the role of FGB gene polymorphisms in the development of cardiovascular diseases revealed that homozygous allelic variant FGB:-455 AA, as well as FGB: –148C/T gene polymorphism (TT genotype) all have great clinical significance [80].

Interestingly, in contrast to the groups stated above, the presence of the same allelic variants of the FGB gene does not lead to intense increase in the level of fibrinogen in blood plasma in the Japanese and indigenous people of North America. Moreover, initial fibrinogen level with GG genotype in these populations is significantly lower, as well as the risk of cardiovascular pathologies [63].

Regarding the frequency of ITGA2 gene variants in different populations, studies in recent years consider this issue in the context of association of several polymorphisms (ITGA2 807C>T, ITGA2 873G>A, ITGA2 1648 G>A) in different ethnic groups. Some major haplotypes are highlighted: ITGA2 807C/1648A haplotype is most common in African populations. It causes reduced expression of receptor and glycoprotein Ib variant (GPIb) – T-to-C substitution at position -5 (ATG:-5T/C, or Kozak element). This substitution leads to disorder in the regulatory sequence and triggers thrombosis. This significantly distinguishes this population from European, Asian and South American populations.

For European and Asian populations, the ITGA2 807T variant plays a key role in development of thrombotic risk and aspirin insensitivity regardless of combinations with other polymorphic variants. It was shown that this polymorphism is found in 40.8 % of patients with resistance to antiplatelet therapy in European sample and 58.6 % of patients of Asian population (China) [142].

Regarding coagulation factors F7 and F13, it was established that their genes are characterized by a high degree of polymorphism in all population studies.

Gene variants F7 R353Q (F7: 10976 G>A) and F7:402G/A are the most common in European and Asian populations. In this case, there is a general trend within separately taken populations and ethnic groups: variant F7:402G/A increases thrombogenic risk, whereas F7: 10976 G>A is characterized by protective activity against development of cardiovascular events and thrombogenic risk. Variant F7: 10976 G>A is the most common among European population, and slightly less common in Asian and African populations [67, 115].

Examination of F13 gene polymorphisms revealed different allelic variants in the sequences encoding subunits A and B (F13A1 and F13B), including: F13A1 Val34Leu, F13A1 Tyr204Phe, F13A1 Pro564Leu and F13B His95Arg, which have in common reduced risk of ischemic stroke and thrombotic events. Here, F13A1Val34Leu is the most common variant in all populations, except Asia (0.6 %): Europeans – 25 %, African population – 18.1 % [85, 147].

Indications for genetic analysis:

- Cases of hereditary thromboembolism in the family;
- Cases of thrombosis in history:
  - Single thrombosis before the age of 50;
  - Repeated thrombosis;
  - Thrombosis at any age if there is a family history;
  - Thrombosis of unusual localization (portal, mesenteric and cerebral vein thrombosis);
  - Thrombosis of unclear etiology after the age of 50;
- *Use of hormonal contraception or hormone replacement therapy* in women who have a history of thrombosis or first-degree relatives diagnosed with hereditary thrombophilia or family history of thromboembolic complications;
- *Complicated obstetric history;*
- *Women planning pregnancy*, who have a history of thrombosis or first-degree relatives diagnosed with hereditary thrombophilia or family history of thromboembolic disease;
- *High-risk situations:*
  - Massive surgical interventions;
  - Prolonged immobilization;
- *Prevention of thrombotic complications in patients with malignancies*.

DNA-Technology developed a kit for identification of polymorphisms associated with thrombophilic risk using real-time PCR technique (see Tables 54 and 55).

Table 54. Genetic polymorphisms associated with thrombophilic risk

Gene	Function of gene product	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
F2 – prothrombin (coagulation factor I)	Prothrombin – the precursor of thrombin (a protein that stimulates blood clot formation).	**20210 G>A**	**rs1799963**	**G/G**	No abnormalities
				G/A	Increased gene expression. Plasma prothrombin levels increased by 30 %, which leads to increased thrombin formation and causes high risk of thrombosis. Early reproductive losses
				A/A	Increased gene expression. Plasma prothrombin levels increased by 70 %, which leads to increased thrombin formation and causes extremely high risk of thrombosis. Early reproductive losses
F5 – proaccelerin (coagulation factor V)	Factor V, together with other factors, forms a complex called prothrombinase, which converts prothrombin to thrombin.	**1691G>A (Arg506Gln)**	**rs6025**	**G/G**	No abnormalities
				G/A	Activated protein C resistance, which normally breaks down activated factor V, thereby preventing uncontrolled expansion of blood coagulation (APC resistance). Recurrent miscarriage, late fetal loss. For homozygotes, the risk of venous thrombosis is increased by 50-100 times
				A/A	
F7 – proconvertin or convertin (coagulation factor VII)	In active state, factor VII interacts with factor III, which leads to activation of factors IX and X of the coagulation system, i.e. coagulation factor VII is involved in blood clot formation.	**10976 G>A (Arg353Gln)**	**rs6046**	**G/G**	No abnormalities
				G/A	30% reduction in factor VII expression in the blood, reduced risk of myocardial infarction, more severe haemophilia
				A/A	50% reduction in factor VII expression in the blood, reduced risk of myocardial infarction, more severe haemophilia
F13A1 – fibrinase (coagulation factor XIII)	Is involved in formation of insoluble fibrin, which promotes blood clotting. Stabilization of fibrin clot consists both in increase in its mechanical strength and protection against lysis.	**103 G>T (Val35Le)**	**rs5985**	**G/G**	No abnormalities
				G/A	Reduced plasma concentrations of factor XIII, disorders in the structure and properties of fibrin clot, which can be a cause of delayed hemorrhage
				A/A	

Gene	Function of gene product	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
FGB – fibrinogen (coagulation factor I)	Under the influence of thrombin, insoluble protein fibrin is formed from fibrinogen in the final blood coagulation phase	**-455 G>A**	**rs1800790**	**G/G**	No abnormalities
				G/A	Constant increase in gene expression, resulting in 10-30% increase in fibrinogen levels in the blood Cardiovascular diseases
				A/A	
ITGA2 – integrin, α2 (platelet collagen receptor)	Ensures interaction of platelets with damaged vessel wall, which activates a cascade of reactions involving coagulation factors	**ITGA2: 807 C>T (F224F)**	**rs1126643**	**C/C**	No abnormalities
				C/T	Changes in the primary structure of the subunit lead to changes in the properties of receptors and marked increase in platelet adhesion
				T/T	
ITGB3 – integrin, b3 (platelet fibrinogen receptor)	Is involved in platelet aggregation, largely responsible for platelet adhesion to subendothelial structures.	**1565 T>C (L33P)**	**rs5918**	**T/T**	No abnormalities
				T/C	Increased affinity to fibrinogen, increased cell adhesion, more intense fibrin clot retraction
				C/C	
PAI-1 – serpin (tissue plasminogen activator inhibitor)	The main function – limitation of fibrinolytic activity at the location site of haemostatic plug through inhibition of tissue plasminogen activator.	**-675 5G>4G**	**rs1799889**	**5G/5G**	No abnormalities
				5G/4G	Slight increase in PAI-1 levels in the blood, decreased blood fibrinolytic activity
				4G/4G	Increased PAI-1 levels, decreased blood fibrinolytic activity. Decreased probability of embryo implantation during IVF Increased risk of thrombosis in protein S deficiency

** Labeling in the dbSNP database of the National Center for Biotechnological Information, NCBI*

Table 55. Thrombophilia Susceptibility Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (192 mcl)
Mineral oil	1 vial (7,68 ml)
PCR buffer	1 vial (3,84 ml)
Polymorphisms studied	F2: 20210 G>A – 960 mcl F5: 1691G>A – 960 mcl F7: 10976 G>A – 960 mcl F13: 103 G>T – 960 mcl FGB: -455 G>A -960 mcl ITGA2: 807 C>T – 960 mcl ITGB3: 1565 T>C – 960 mcl PAI-1:-675 5G>4G – 960 mcl
Specimen for analysis	Whole blood

Technology:
- PCR melting;
- Use of other technological platforms is not permitted.

Storage temperature:
+2 to +8 °C (-20 °C for Taq-AT-polymerase).

Equipment required for analysis:
DT devices produced by DNA-Technology: DTlite, DT-prime.

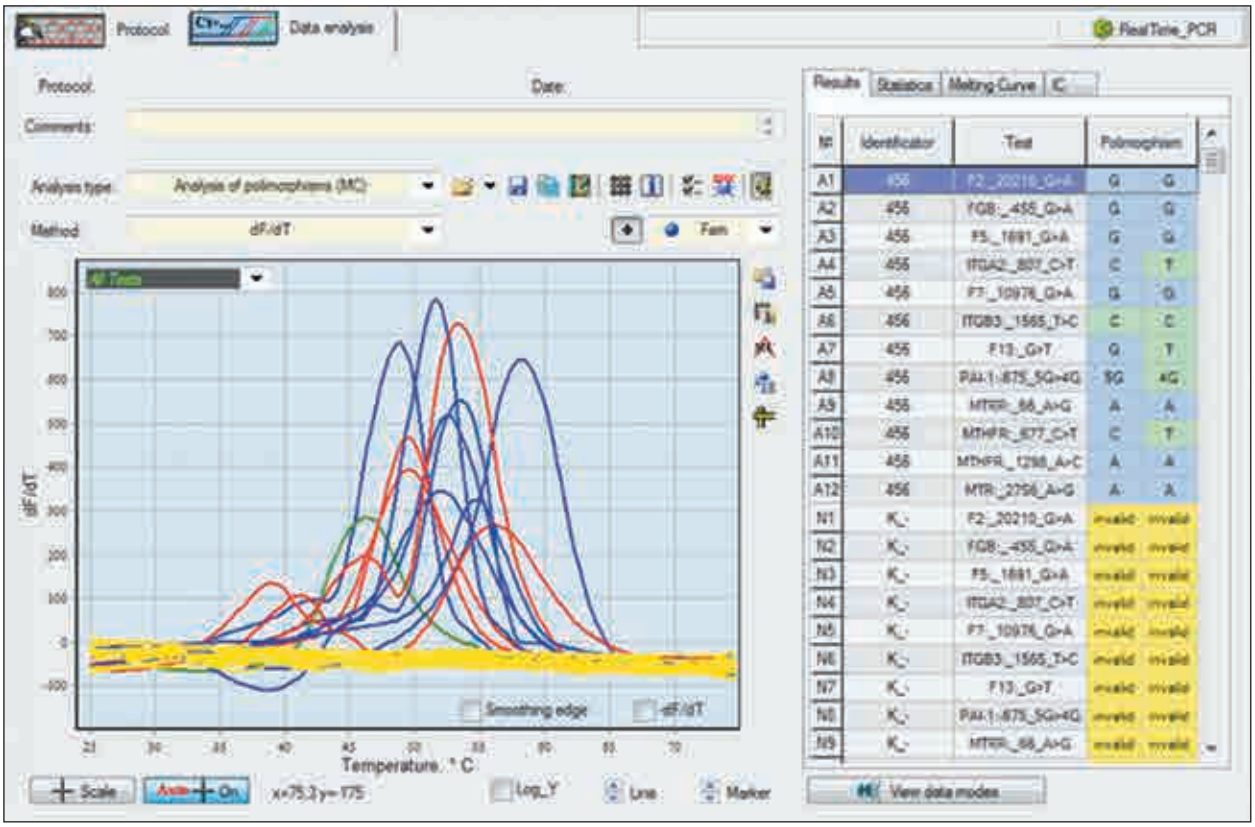
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

Minimum amount of DNA for analysis:
1.0 ng for amplification tube.

The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

Software:
Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 31).

A



B

№	Name of research	Results	
		Genotype	
1	F2:_20210_G>A	G	G
2	FGB:_-455_G>A	G	A
3	F5:_1691_G>A	G	A
4	ITGA2:_807_C>T	C	C
5	F7:_10976_G>A	G	A
6	ITGB3:_1565_T>C	T	T
7	F13:_G>T	G	G
8	PAI-1:_-675_5G>4G	5G	4G
9	MTRR:_66_A>G	A	G
10	MTHFR:_677_C>T	C	C
11	MTHFR:_1298_A>C	A	A
12	MTR:_2756_A>G	A	A

Fig. 31. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4.4. Folate Metabolism Real-Time PCR Genotyping Kit

Folic acid (vitamin B9) is a water-soluble vitamin, required for the growth and development of the circulatory and immune systems. Folic acid deficiency can cause megaloblastic anemia in adults and can increase the risk of neural tube defects during pregnancy. Folic acid is called **folate**.

Humans and animals do not synthesize folic acid, receiving it mainly together with food. Folic acid is found in large quantities in green leafy vegetables, legumes, whole wheat bread, yeast and liver. The laws in many countries require manufacturers of flour products to enrich grains with folic acid [129].

A group of folate compounds plays a leading role in a wide range of vital processes:

- Stimulates erythropoiesis;
- Involved in synthesis of amino acids, nucleic acids, purines, pyrimidines and vitamins;
- Involved in choline and histidine metabolism;
- An important contributing factor in DNA and RNA methylation;
- Promotes regeneration of muscle tissue;
- Influences the development of fast-growing tissues (skin, gastrointestinal tract cover, bone marrow);
- Protects the fetus during pregnancy against the action of teratogenic and damaging factors;
- Promotes normal maturation and functioning of the placenta;
- Folic acid has estrogen-like activity that helps to reduce intake of hormones during hormone replacement therapy.

These functions are realized during folate metabolism, which forms the basis of the folate cycle (Fig. 32).