DNA-TECHNOLOGY

CATALOG
PCR KITS

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PCR KITS

Moscow
2019

TABLE OF CONTENTS

NOTE! **The information contained in this catalog may not be consistent with the latest version of the specifications for the indicated product**

ABBREVIATIONS

AASLD American Association for the Study of Liver Diseases
ABCB1 ATP-binding cassette, sub-family B
ACS Acute Coronary Syndrome
ADP Adenosine Diphosphate
AIDS Acquired Immune Deficiency Syndrome
ARVI Acute Respiratory Viral Infections
ASA Acetylsalicylic Acid
BGC Bacterial Genome Count
BMD Bone Mineral Density
cDNA Complementary DNA
CFTR Cystic Fibrosis Transmembrane conductance Regulator
CHEK2 Cell cycle heckpoint kinase 2
CHC Chronic Hepatitis C disease
CMV Cytomegalovirus
CNS Central Nervous System
COL1A1 Collagen, type I, alpha 1
COPD Chronic Obstructive Pulmonary Disease
CTR Calcitonin Receptor
CVD Cardiovascular Disease
DBP Vitamin D-binding Protein
DNA Deoxyribonucleic Acid
dATP deoxyadenosine triphosphate
dGTP deoxyguanosine triphosphate
dNTP deoxynucleotide triphosphate
dTTP deoxythymidine triphosphate
dCTP deoxycytidine triphosphate
EAA European Academy of Andrology
EASL European Association for the Study of the Liver
EBV Epstein-Barr virus
EDIs Especially Dangerous infections
ER Estrogen Receptor
ESR1 Estrogen Receptor1
FDA Food and Drug Administration – (a US federal agency responsible for protecting and promoting public health through regulation and supervision of food safety, pharmaceutical drugs and medical devices.)
FLASH Fluorescent Amplification-based Specific Hybridization
GBS Group B Streptococcus
GE Genome Equivalent
GMF Genetically Modified Food
GMO Genetically Modified Organism
GR Glucocorticoid Receptor
HBV Hepatitis B Virus
HCV Hepatitis C Virus
HER Human Epidermal Growth Factor Receptor 2
HHC Hereditary Hemochromatosis
HHV Human Herpes Virus
HIV Human Immunodeficiency
HLA human leucocyte antigens
HPV Human Papillomavirus
HSV Herpes Simplex Virus
ICD International Classification of Diseases
ICSI Intracytoplasmic Sperm Injection
IHD Ischemic Heart Disease
IHR International Health Regulations
IL Interleukin
INR International Normalized Ratio
IRT Immunoreactive Trypsin
IU International Unit
IVD *In vitro* diagnostics
IVF *In vitro* fertilization
MESA Microsurgical Epididymal Sperm Aspiration
NIBSC The National Institute for Biological Standards and Control
PBM Peak Bone Mass
PCR Polymerase Chain Reaction
PESA Percutaneous epididymal sperm aspiration
PID Pelvic Inflammatory Diseases
PII Percutaneous Intracoronary Intervention
RNA Ribonucleic Acid
PR Progesterone Receptor
PTH Parathyroid Hormone Receptors
qPCR Quantitative real time polymerase chain reaction
RANKL Receptor Activator of Nuclear Factor kappa-B Ligand
RAS Renin-Angiotensin System
RUO Research Use Only
RT-PCR Real-Time Polymerase Chain Reaction
RT Reverse Transcriptase
Rt Real Time
SARS Severe Acute Respiratory Syndrome
SCO Sertoli Cell-only Syndrome
SIC Sample Intake Control
SNP Single Nucleotide Polymorphism
SoGAT Standardization of Genome Amplification Techniques
SpA Seronegative Spondyloarthritis
STIs Sexually Transmitted Infections
STS Sequence-Tagged Site (short unique DNA sequences that can be amplified in the presence of other genomic DNA sequences)
SVR Sustained Virologic Response
TESA Testicular Sperm Aspiration
TESE Testicular Sperm Extraction
VDR Vitamin D Receptor
VZV Varicella Zoster Virus
WHO World Health Organization

THE TERMS USED

Allele (*from the Greek word 'allelon', meaning "of each other"*) refers to different forms of the same gene located in the same place (*locus*) on homologous chromosomes, which controls alternative variants of the same sign. All the genes of somatic cells, except for the genes located in the sex chromosomes, are represented by two alleles, one inherited from the father, and the other from the mother.

Gene (*from the Greek word 'genos', meaning birth, origin, race, species, or class*) is the material carrier of genetic information, which is a section of DNA, carrying the complete information about the structure and characteristics of the synthesis of one protein molecule.

Genetic polymorphism is the coexistence within a population of two or more different inherited forms for whose gene portion in a population there is more than one variant of nucleotide sequence. The most common is *single-nucleotide polymorphism (SNP)* – substitution of one nucleotide for another at a particular point of the genome.

Genotype *(from Greek words 'genos', which means birth or origin and 'typos' which means a mark)* is a set of alleles of a gene or group of genes controlling the analyzed traits in a given organism.

Heterozygous genotype (heterozygous state of a gene) is a genotype containing different alleles of one gene.

Hyperergia *(from Greek words 'hyper' meaning over, and 'ergon' meaning work)* refers to increased reactivity.

Homozygous genotype (homozygous gene) is a genotype containing identical alleles of one gene.

Multifactorial diseases (diseases with hereditary component) are diseases, which develop from interaction of certain genetic factors and specific impacts from environmental factors.

Nucleotide is a complex chemical group found in a natural state. It consists of a nitrogenous base, linked to a sugar, and phosphoric acid. It is single unit of nucleic acid (DNA and RNA) molecule. There are four types of nucleotides that make up the nucleotide sequence: **A** (adenine), **G** (guanine), **T** (thymine), **C** (cytosine) – the DNA sequence; **A** (adenine), **G** (guanine), **T** (thymine), **U** (uracil – not found in DNA; thymine is replaced by uracil in RNA) – the RNA sequence.

Positive predictive value is the probability of having a disease with a positive (pathological) test result.

Risk factors – the general name of factors that are not the direct cause of a specific disease, but make a person more likely to develop the disease. They are divided into modifiable (behavioral) and non-modifiable (physiological) risk factors.

Phenotype *(from Greek words 'phainon', which means revealing and 'typos', which means mark)* designates the totality of manifestations of a genotype (the overall appearance of the body), and in the narrow sense – an individual's observable traits (phenes), controlled by certain genes. The concept 'phenotype' applies to any traits of the body, starting from the primary products of gene action (RNA and polypeptide molecules) and ending with characteristics of the external structure, physiological processes, behavior, etc. The phenotype is formed based on interaction of the genotype and a number of environmental factors.

OR (odds ratio) is the ratio of chances. It is defined as the chances of an outcome under the influence of a risk factor divided by the chances of that outcome without the influence of a risk factor. In this case, it is used to estimate the chances of developing a clinical condition depending on the genotype of an individual. OR> 1 corresponds to an increased risk of developing the condition being analyzed, while OR<1 corresponds to a decrease in the risk.

ABOUT THE COMPANY

DNA-Technology is a unique company for Russian biotech business environment due to its full-cycle R&D and manufacturing process.

The company has been developing, producing and introducing to laboratories high-tech equipment and kits for PCR assays since 1993.

Our team brings together leading experts in the field of molecular biology, immunogenetics, medicine, thermodynamics, optics, electronics and programming. Implementing skills they form technological and scientific potential of the company, that allows providing a high standards and quality control of production at all stages.

DNA-Technology production sites meet all the modern requirements for medical equipment and PCR kits production. This is evidenced by the license issued by the Federal Service on Surveillance in Healthcare and Social Development of Russian Federation and the certificates verifying the fact that the quality management system is complied with ISO 9001:2015 and ISO 13485:2016.

THE MAIN DIRECTIONS OF «DNA-TECHNOLOGY» ACTIVITIES:

- Customers support at any level of PCR laboratory workflow: designing a plan for laboratory facilities, supplying kits, equipment and consumables, personnel training, analyzing the results of genetic tests and interpreting them for clinicians.
- Developing and manufacturing of equipment and software for PCR analysis in the scientific and clinical fields.
- Producing a wide range of kits for clinical bacteriology, virology, genetic diagnostic and detection of DNA of infectious agents in agricultural crops.
- Providing service support.
- Joint projects with clinicians and researchers.

Our product range includes basic equipment and devices for PCR laboratories:

- Detecting thermocyclers for real-time PCR analysis (*DT* devices);
- Thermostats;
- Power supplies;
- PCR cabinets.

The company has a strong R&D department for developing high-sensitivity kits for PCR analysis, such as:

- detection of viral and bacterial infections:
  - Hepatitis and HIV;
  - Urogenital infections;
  - Herpesvirus infections;
  - Human papillomavirus infections;
  - Respiratory tract infections;
  - Especially dangerous and natural focal infections;
  - Other infections;
- Analysis of microbiome composition of the urogenital tract;
- Molecular Genetics.

The company provides unique technologies for diagnostic of genetic predisposition to disorders such as polyorganic pathologies, oncology diseases, disorders of metabolic processes, malfunctioning of immune system and more.

Our goal is to apply the latest scientific findings in the field of molecular biology methods to routine laboratory practice. We do our best to improve diagnostic quality in order to make the treatment more effective and the prognosis of clinical outcome more precise.

I. PCR ADVANTAGES

PCR method is one of the most rapidly developing field in molecular genetic analysis. Extensive use of PCR technology has made it possible to significantly improve the quality of laboratory analysis and to reduce time of analysis.

Nowadays a modern PCR laboratory provides an opportunity to carry out multifactorial, multiplex and quantitative analysis by detecting nucleic acids of various microorganisms in the given sample.

Real-time PCR method makes it possible to detect the presence of an infectious agent (qualitative analysis) and DNA/cDNA concentration in the sample (quantitative analysis) in the diagnosis of infectious diseases.

Qualitative analysis is used to detect non-opportunistic pathogens, such as gonorrhea, chlamydia and trichomonas.

However, the situation for opportunistic pathogens is quite different – qualitative analysis is not sufficient for this group of microorganisms, as the term "opportunistic" itself implies that the only fact of their presence does not mean the presence of a disease. And it is important not only to detect the presence of the microorganisms in a sample, but their concentration as well. In this case, it is reasonable to apply quantitative real-time PCR analysis. Moreover, quantitative analysis is recommended for determining viral load and treatment for viral hepatitis B and C, and HIV.

It is important to note that in some cases it is vital to make a diagnosis within a short period of time with high degree of accuracy in order to avoid development of complications. Applying conventional methods of diagnosis such as culture and microscopic assays do not ensure proper diagnosis and high reliability due to objective reasons.

For example, the main objectives of microscopy are:

- Detection of a causative agent in the clinical material;
- Tentative identification based on the detection of morphological and tinctorial marks of microorganisms;
- Analysis of pure culture.

This method is considered as the fastest and cheapest and its use is associated with minimum requirements for laboratory's establishment.

However, there are a number of limitations in using microscopy for infectious diseases diagnosis:

- Low sensitivity;
- Subjectivity of the results assessment;
- Limited range of the morphotypes detected;
- Approximate quantitative assessment.

The culture method, alongside with microscopic examination of microorganisms, is included in the "golden standard" for diagnostics and allows:

- To detect all living cultivated organisms;
- To determine the antibiotic resistance of microorganisms detected.

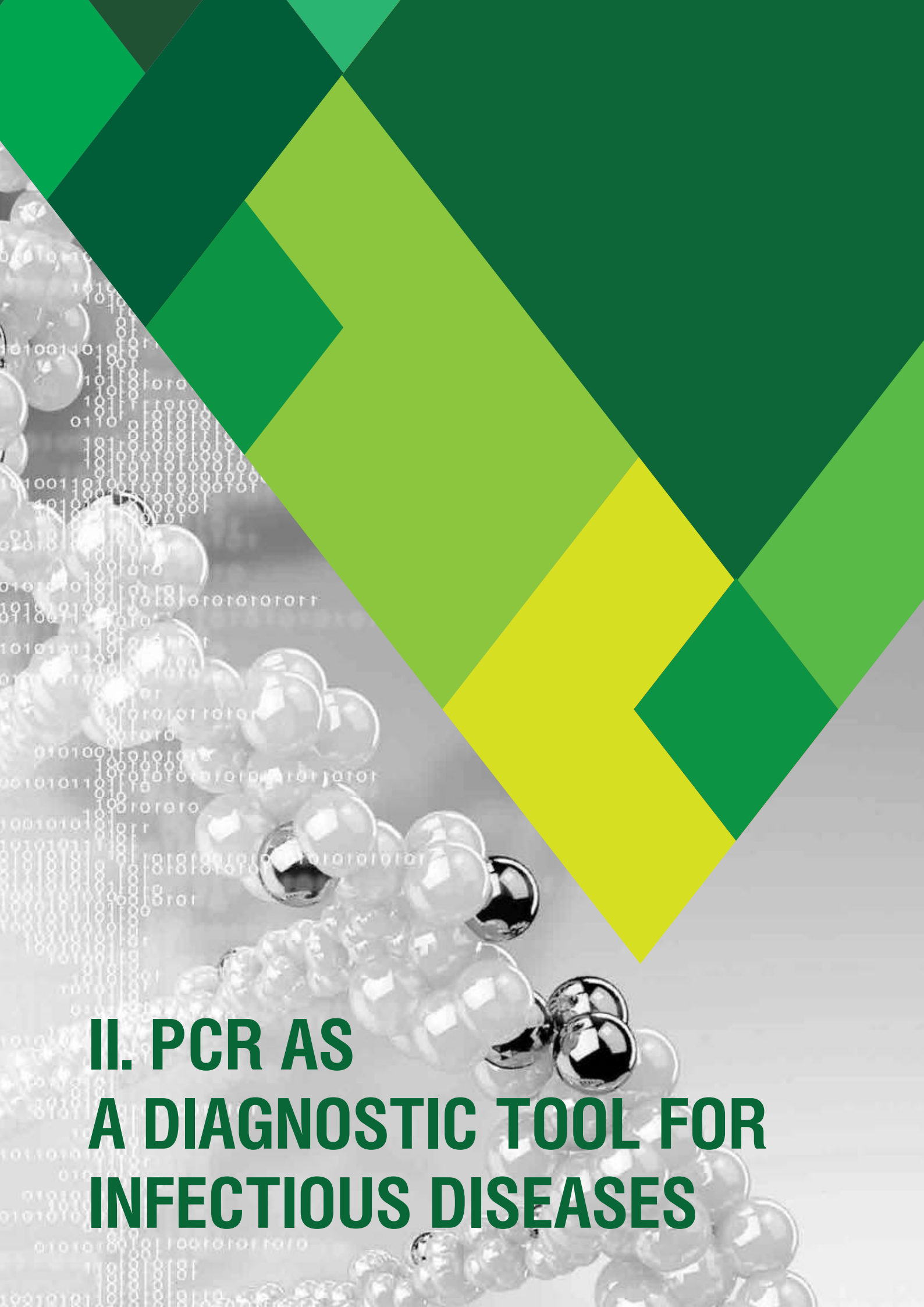
However, the culture method has objective limitations:

- Long cultivation time – from 5 days to 2 months;
- More stringent requirements for biomaterial transportation and storage ;
- Inability to culture most of anaerobic microorganisms;
- More stringent requirements for laboratory's establishment.

Introducing PCR methods to laboratory practice provides modern laboratories with certain important advantages, such as:

- **High-speed analysis** – even the most complex multi-parameter assays take less than one day from the moment the sample is collected to the moment the results are obtained;
- **Determination of microorganisms concentration in a biomaterial;**
- **High reproducibility of results;**
- **Maximum sensitivity and specificity;**
- **Standardized technological process;**
- There is an opportunity for a wide range of infectious agents to be diagnosed from only one sample of biomaterial (when applying other methods it may be required to collect a sample from a patient several times, which may be traumatic and that's why unwelcome);
- Results can be obtained irrespective of the infectious process;
- A patient can be examined regardless of antibiotics treatment;
- Determining genetic factors of microorganisms' antibiotic resistance (options for the most effective therapeutic regimen to be selected).

The important aspect of PCR diagnostics is possibility to conduct genetic analysis, as well as to determine risks of somatic pathologies behaviour (such as thrombophilia) and drug tolerance.



II. PCR AS A DIAGNOSTIC TOOL FOR INFECTIOUS DISEASES

II. PCR AS A DIAGNOSTIC TOOL FOR INFECTIOUS DISEASES

1. OPPORTUNISTIC AND NON-OPPORTUNISTIC PATHOGENS OF THE HUMAN UROGENITAL TRACT

The main causative agents of urogenital infections (sexually transmitted infections) are: *Treponema pallidum, Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis and Mycoplasma genitallium*. The role of these infectious agents in the development of urogenital tract diseases has been proven and now their diagnosis via qualitative methods is beyond question.

Mycoplasma hominis, Ureaplasma urealyticum and *Ureaplasma parvum* can be present in the urogenital tract together with non-opportunistic pathogens. These microorganisms are opportunistic organisms capable of causing a disease when exposed to a variety of endogenous and exogenous factors. At present the role of these microorganisms in disease development is controversial and requires further clarification.

Experts from the World Health Organization (WHO) in 2006 identified *U.urealyticum* as a potential pathogen of non-specific nongonococcal urethritis in men and possibly of pelvic inflammatory diseases (PID) in women. Experts from the US Centers for Disease Control and Prevention (CDC), 2010) do not accept the etiological role and clinical significance of genital mycoplasmas (except for *M.genitalium*) as established.

At the present stage, urogenital tract infections do not have pathognomonic symptoms and they proceed with latent, very mild clinical manifestations, with prevalence of the disease chronic forms. This results in complications such as pelvic inflammatory diseases, reproductive disorders, pregnancy complications, etc..

In this regard, *molecular biological techniques*, primarily PCR method, are becoming the most relevant in STIs diagnostics.

PCR method enables to:

- Identify the etiology of the infection;
- Maintain control over the infection;
- Assess the relative (compared with normal flora) amount of microorganisms, which is important for opportunistic pathogens that cause pathology only under certain conditions (increased concentration due to lower amount of normal flora);
- Evaluate treatment effectiveness.

Among all the undeniable merits of PCR in diagnostics of infectious agents, two of them stand out: ability to detect etiologic agents of the disease in introduction of mixed infection (conducting multiplex analysis) and low traumatism rate when sampling biomaterial is taken for examination.

The high sensitivity and specificity of PCR makes this technique appropriate for STIs diagnosis. In accordance with CDC Guidelines, European guideline for the management of Chlamydia trachomatis infections, and Treatment Guidelines for sexually transmitted diseases, only PCR-based diagnostics can be recommended for *C. trachomatis* detection.

Subjects of screening for STIs are:

- Persons that had sexual contact with STIs patients;
- Persons who are undergoing screening for other STIs;
- Persons from decreed group – during mandatory (at employment) and periodic medical examinations in accordance with approved regulations;
- Women with muco-purulent cervical discharge and adnexitis symptoms;
- Newborns from mothers who have had STIs during pregnancy;
- Sexual partners undergoing preconception checkup;
- Pregnant women;
- Women awaiting pelvic surgery;
- Sexually abused persons;
- Men with mucopurulent urethral discharge and dysuria symptoms;
- Egg and sperm donors;
- Persons under age of 25, who have numerous sex partners;
- Women with infertility, recurrent miscarriage and premature birth history.

Technique for collecting clinical specimen for PCR method:

- Epithelial cell scrapings are used as the specimen.
- The clinical material is obtained using a disposable sterile instrument, such as Cytobrush or scrape for DNA analysis. ***Use of brushes during pregnancy is contraindicated!***
- Before obtaining clinical specimen, freely flowing discharge is removed with a sterile cotton swab.
- Epithelial cell scrapings are obtained by rotating the probe.
- The obtained specimen is put into a tube, such as Eppendorf tube, that contains transport medium.
- The tube with the biomaterial is stored and transported at a household refrigerator temperature (+4 °C) without freezing, for not longer than 1 day.

Clinical specimen may be collected from the following potential infection sites:

Urethra:

Before obtaining the biomaterial, it is recommended to delay urination for at least 1.5 hours;
To obtain a clinical specimen from the urethra, a swab is inserted into male 2-4 cm deep and into female – 1.5-2 cm;
In case of abundant purulent discharges, the clinical specimen should be obtained after urination.

Vagina: a clinical specimen is obtained from the posterior or posterolateral vaginal wall

Endocervical canal: to obtain a clinical specimen, the swab is inserted up to 1.5-2 cm deep.

Rectal ampulla: a clinical specimen is obtained by moving the swab in a circular motion from all over the walls. The swab is inserted up to 3-4 cm deep.

Conjunctiva: a clinical specimen is obtained from the surface of the inferior eyelid conjunctiva moving from the outer to the inner corner of the eye.

Nasopharynx: a clinical material is obtained from posterior pharyngeal wall above the bottom edge of the soft palate and from the surface of the tonsils.

DNA-Technology offers the following kits (see Table 1) for detecting urogenital infections by PCR method.

Table 1. Kits produced by DNA-Technology for detecting urogenital infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Chlamydia trachomatis	*	*	*	–	RU/IVD
Mycoplasma hominis	*	*	*	–	CE/IVD
Mycoplasma genitalium	*	*	*	–	CE/IVD
Ureaplasma complex (U.urealyticum/ U.parvum)	*	*	*	–	CE/IVD
Ureaplasma parvum	*	*	*	–	CE/IVD
Ureaplasma urealyticum	*	*	*	–	CE/IVD
Trichomonas vaginalis	*	*	*	–	CE/IVD
Gardnerella vaginalis	*	*	*	–	CE/IVD
Neisseria gonorrhoeae	*	*	*	–	CE/IVD
Candida albicans	*	*	*	–	CE/IVD
TNC multiplex *(T.vaginalis/ N.gonorrhoeae/ C.trachomatis)*	–	–	*	–	RU/IVD
UMC multiplex *(U.urealyticum; parvum/ M.genitalium/ C.trachomatis)*	–	–	*	–	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

Kit format:
- Strip tubes (8 pcs., 0.2 ml each);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C.

Shelf life:
- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months (except *TNC multiplex* and *UMC multiplex* kits – 6 months).

DNA extraction kits of reagents:
- *PREP-RAPID;*
- *PREP-NA;*
- *PREP-GS.*

Specimen for PCR testing:
- Scrapings from mucosa ;
- Urine cell sediment.

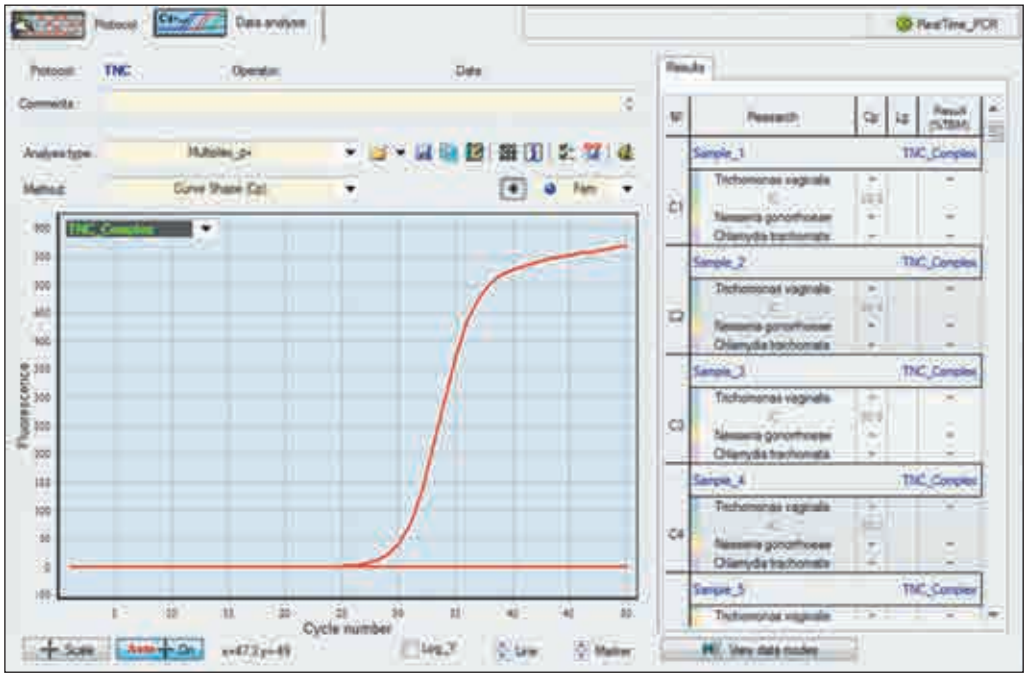
Recommended additional reagents:
SIC – sample intake control – is designed for DNA identification (qualitative analysis) and approximate estimation of the amount of human genomic DNA (quantitative analysis) in a human biomaterial by real-time PCR.

Equipment required for analysis:
- For FLASH kits: Gene, Gene-4 or counterparts
- For Rt kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories and Rotor-Gene devices produced by QIAGEN (except for *TNC multiplex* and *UMC multiplex* kits).

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 1).

A



B

№	Name of research	Results
1	Trichomonas vaginalis	not discovered
2	Neisseria gonorrhoeae	not discovered
3	Chlamydia trachomatis	+

Fig. 1. Results of *Rt* qualitative analysis (DT devices) TNC Multiplex REAL-TIME PCR Detection Kit
A – Optical measurement analysis (Fam channel)
B – Analysis results

2. HERPESVIRUS INFECTIONS

Herpesviruses are abundant in nature. Nowadays more than 100 herpes viruses are distinguished, a small amount of which occurs in humans.

Herpesvirus can remain persistent in a human body for the entire lifetime causing diseases with multiple clinical implications (see Table 2).

Table 2. Diseases caused by herpesviruses

Type of herpesvirus	Diseases caused
Labial herpes simplex virus (type 1)	Herpes simplex: lesions of skin, mouth mucous membrane, conjunctiva or cornea, encephalitis
Genital herpes simplex virus (type 2)	Genital herpes: lesions of genitals mucous membranes, central nervous system damage in newborns
Varicella zoster virus (type 3)	Chickenpox, herpes zoster, postherpetic neuralgia, necrotizing herpes zoster in HIV infection
Epstein-Barr virus (type 4)	Infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal cancer, genital ulceration
Cytomegalovirus (type 5)	Cytomegalovirus infection: neonatal pathologies, transplantation complications, such as interstitial pneumonia, gastrointestinal disorders, hepatitis
Herpesvirus (type 6)	Infectious roseola (exanthem subitum), interstitial pneumonia
Herpesvirus (type 7)	Chronic fatigue syndrome, exanthem subitum
Herpesvirus (type 8)	Kaposi sarcoma

Herpes simplex virus (HSV), Type 1 and 2.

Herpes simplex virus invades a human body through damaged skin and mucous membranes. During the first phase of pathogenesis, the virus gets into epithelial cells (mucosa of oral cavity, pharynx or genital organs), where its replication occurs. Papules and vesicles, characteristic for herpes infection, appear on mucosa and skin.

Genital herpes (GH) is a herpes simplex virus. It remains persistent in men in the urogenital tract, and in women in the cervical canal, vagina and urethra. Infecting with genital herpes occurs mainly through sexual contact. During viremia in pregnant women, a fetus is infected via hematogenic route, and during childbirth via contact. Herpes infection arising from viremia may result in afflicting several organs at a time.

Herpetic esophagitis, pneumonitis and hepatitis can develop. Damage to peripheral nervous system can occur in the form of ganglionitis, ganglionevrita, radiculoneuritis and polyneuropathy. Central nervous system (CNS) damage with herpes infection most commonly proceeds as encephalitis or meningoencephalitis.

Specimen for PCR testing:

- Vesicles fluid;
- Scrapings from mucosa ;
- Blood;
- Mucus;
- Urine;
- Lacrimal fluid;
- Cerebrospinal fluid.

PCR specimen is taken from eruption sites only during the period of acute infection.

Cytomegalovirus (CMV)

The virus invades a body through saliva, genital secretions during sexual intercourse, through breast milk, during organ transplantation and donor blood transfusion, when using donor sperm and ovigerms, as well as non-sterile syringes. Moreover, introduction of cytomegalovirus (CMV) infection can occur even during fetus prenatal development period in pregnant women with viremia.

CMV infection mostly manifests itself as:

- ARVI (acute respiratory viral infection). Complaints of weakness, general malaise, rapid fatigability, headache, runny nose, sialadenosis, saliva flux and whitish film on gums and a tongue.
- Generalized CMV infection with visceral injury, inflammatory disorder of:
  - Hepatic tissue;
  - Adrenal glands;
  - Spleen;
  - Pancreatic glands;
  - Kidney.

This is accompanied by frequent pneumonia and bronchitis that poorly respond to antibiotic therapy. Lesions of eye vessels, intestinal wall, brain and peripheral nerves, as well as enlarged parotid and submandibular salivary glands, arthritis, and skin rash are rather common.

- Lesion of urogenital organs in men and women manifests itself by symptoms of chronic non-specific inflammation.
- Pathologies in pregnancy, fetus and newborn result in progress of serious illness and central nervous system damage (mental developmental delay, hearing loss). In 20-30 % of the cases a baby dies.

Specimen for PCR testing:

- Urethral scrapings;
- Cervical and vaginal scrapings;
- Cell urocheras;
- Blood;
- Peripheral blood mononuclear cells;
- Cerebrospinal fluid.

Varicella Zoster Virus (VZV, HHV-3)

VZV causes two types of lesions: chickenpox and herpes zoster. The main infection transmission routes are airborne and non-percutaneous channels of infection (through discharge of vesicular fluid).

VZV is a causative agent of chicken pox in children. It invades a body during the childhood and remains in sensory cerebrospinal and cerebral ganglions in a latent form. With immunity deterioration the virus becomes active and causes radiculitis. In some cases retrograde spread of the virus in the CNS via sensitive pathways occurs.

Vesicular rashes appear on the skin of patients suffering from herpes zoster. Vasculitis, mostly observed after eye-lesion, can indicate that the virus has invaded CNS. VZV often affects HIV-infected people at different stages of the disease. Apart from herpes zoster, it can cause damage to the nervous system, such as encephalitis and myelitis.

Specimen for PCR testing:

- Vesicles fluid or rash scrapings;
- Scrapings from mucosa;
- Blood;
- Cerebrospinal fluid.

Epstein-Barr Virus (EBV, HHV-4)

Human is the only reservoir of infection. The virus is mainly transmitted by air; less frequently it is vector-borne or through sexual intercourse.

At an early age, the infection is accompanied by slight manifestations or it is generally asymptomatic; initial infection at teenage or more advanced age can cause a disease known as infectious mononucleosis. Chronic EBV infection is more common among patients with immunodeficiency. Chronic infection mostly manifests itself in the form of progressive lymphoproliferative disease or CNS lymphoma.

The virus can cause malignant transformation of cells, that gives reason to assume that it is involved in such diseases as African forms of Burkitt's lymphoma, nasopharyngeal carcinoma in men from some ethnic groups of southern China, as well as Kaposi sarcoma in patients with AIDS.

Specimen for PCR testing:

- Urethral scrapings;
- Blood;
- Peripheral blood mononuclear cells;
- Cerebrospinal fluid.

Herpes Virus Type 6 (HHV-6)

The disease is typically latent in nature, sometimes with clinical implications in the form of sudden exanthema (exanthema subitum, children roseola (sudden) or false rubella – an acute viral infection in young children), a syndrome similar to mononucleosis.

This virus is associated with chronic fatigue syndrome, which manifests itself as an incipience of a respiratory disease, with catarrhal symptoms, fever, sore throat, erratic myalgia, cervical, neck and axillary lymph-node hyperplasia, joint pain, sleep disturbances, muscle weakness, undue fatiguability, and hyperirritability.

The diagnostic criteria of this syndrome include: chronic fatigue and performance decrement by more than 50 % within 6 months in the absence of other diseases (cancer, diseases of liver, kidney, heart, etc.) which cause similar symptoms.

The virus is also associated with B-cell lymphoma. In 80-90 % of cases of B-cell lymphomas, integrated DNA fragments, homological to the virus genome, are detected in the transformed cell, which gives ground to consider it a potential etiologic agent of the disease.

Specimen for PCR testing:

- Blood;
- Urine;
- Phlegm;
- Throat swab.

Human Herpesvirus 8 (HHV-8)

It was found that HHV-8 is associated with all types of Kaposi sarcoma, including endemic African Kaposi sarcoma, classic Mediterranean Kaposi sarcoma among elderly people and transplant-related Kaposi sarcoma. The DNA of this virus is constantly detected in the tissue of AIDS-related Kaposi sarcoma, whereas it is not detected in the normal tissue of neighboring sections.

It was demonstrated that the course of Kaposi sarcoma is preceded by an infection caused by HHV-8. Moreover, the virus is a developing factor for primary lymphoma of serous sac, and some varieties of Castleman's disease.

Specimen for PCR testing:

- Blood;
- Urine;
- Phlegm;
- Throat swab.

DNA-Technology offers the following kits (see Table 3) for detecting herpesvirus infections by PCR.

Table 3. Kits produced by DNA-Technology for detecting herpesvirus infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Herpes symplex virus 1,2	*	*	*	–	CE/IVD
Human herpesvirus 6	*	*	*	–	RU/IVD
Human herpesvirus 8	*	*	*	–	RU/IVD
Cytomegalovirus	*	*	*	–	RU/IVD
Epstein Barr virus	*	*	*	–	CE/IVD
Varicella zoster virus	*	*	*	–	CE/IVD
Herpes multiplex (HSV1/HSV2/CMV)	–	–	*	–	RU/IVD

*** Note:**

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RUO – kits for Research Use Only

Kit format:

- Strip tubes (8 pcs., 0.2 ml each);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C.

Shelf life:

- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months (except for Herpes multiplex reagent kits – 6 months).

DNA extraction kits:
- *PREP-RAPID;*
- *PREP-NA;*
- *PREP-GS.*

Specimen for screening:
- Vesicles fluid;
- Scrapings from mucosa;
- Biopsy specimens;
- Blood;
- Cerebrospinal fluid.

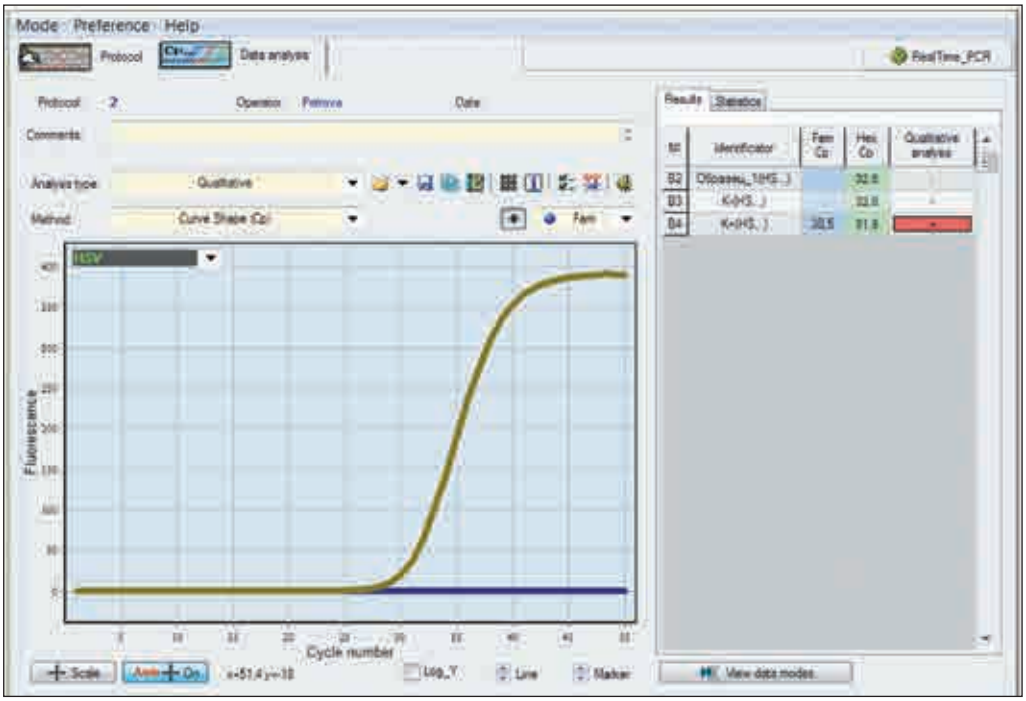
Recommended additional reagents:
SIC – sample intake control – is designed for DNA identification (qualitative analysis) and approximate estimation of the amount of human genomic DNA (quantitative analysis) in a human biomaterial by real-time PCR.

Equipment required for analysis:
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  – DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  – IQ5 Cycler device produced by Bio-Rad Laboratories and Rotor-Gene devices produced by QIAGEN (except reagent kit *Herpes multiplex*).

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 2).

A



B Qualitative analysis

Number of the hole	Identificator of the tube	Cp, Fam	Cp, Hex	Result
B2	Sample_456		32.6	–
B3	K–		32.6	–
B4	K+	30.5	31.9	+

Fig. 2. Results of *Rt* qualitative analysis (DT devices) HSV 1,2 REAL-TIME PCR Detection Kit
A – Optical measurement analysis (Fam channel)
B – Analysis results

3. HUMAN PAPILLOMAVIRUS INFECTIONS

Human papillomavirus (HPV) belongs to the genus of papilloma viruses of the Papovaviridae family and has a definitive biocycle associated with differentiation of keratinocytes. The virus infects only proliferating epithelial cells of the basal layer. Formation of viral particles occurs in the upper layers of the epithelium. Presently over 100 types of HPV are distinguished, with about 30 of them being able to infect the epithelium of a human urogenital tract.

Clinical implications of HPV infection:

- Skin lesions (plantar wart, common wart, flat wart, Butcher's wart, warty epidermodysplasia, Bowen's disease, non-warty skin lesions, and carcinoma).
- Lesions of the mucous genitals (*condylomata accuminata*, giant condyloma (Buschke-Lowenstein tumour), non-condylomatous lesions, carcinoma).
- Lesions of the mucous membranes of other organs (laryngeal papilloma, tonsillar carcinoma, neck carcinoma, tongue carcinoma).

Diseases associated with human papillomavirus infection:

- Anogenital (venereal) warts;
- Viral warts: simple, vulgar;
- Laryngeal papillomatosis;
- Benign neoplasms of male genital organs;
- Cervical neoplasia.

HPV infection route:

- Sexual way, including oral-genital contact and anal sex;
- Vertical transmission – at birth, that causes laryngeal papillomatosis with babies and anogenital warts with infants;
- Domestic path – external manifestations of the virus – pointed condyloma;
- Self-infection (autoinoculation).

Papillomaviruses are divided into two groups based on ability to induce malignant transformation of the epithelium: low-risk and high-risk oncogenic papillomas.

The low-risk oncogenic group includes Types 6, 11, 36, 42, 43, 44, 46, 47 and 50. An infection caused by these types usually occurs in the form of benign cervical lesions and condylomas.

The high-risk oncogenic group includes Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73 and 82. Type 16 is the most common and it is detected in over 50 % of all cervical cancer cases in Europe.

Cervical cancer is the second largest (after breast cancer) malignant tumor of the reproductive system. In the age group from 16 to 40, cervical cancer is the second leading cause of mortality among patients with malignant tumors.

The disease progression from epithelial infection to cervical cancer formation takes at least 5 years (10-20 years on average), and only in very rare cases it can take 1-2 years. That is why a significant role in cervical cancer prevention should be given to screening that would detect the presence of HPV infection and precancerous changes or cancer at the early stages.

The most frequently used test for screening of cervical cancer and precancerous changes in the epithelium is a *cytological* one. At the same time the diagnostic accuracy can vary depending on the specimen collection method, binding technique and smear preparation as well as a researcher's skills. It is believed that nearly one third of cervical cancer cases are diagnosed in women who were screened regularly during cytological examination and thus false negative results for these patients were obtained. Therefore, DNA diagnostics of HPV infection is currently considered the basis for cervical cancer screening and prevention.

The fact of HPV presence can be used for adjusting an examination plan and frequency of monitoring of pacient. The positive test result has high diagnostic significance for women's age over 30. DNA diagnosis is used as a confirmatory test when detecting ASC-US (after liquid cytology and visual method) and when monitoring a therapy for CIN II, III.

DNA-Technology offers the following kits (see Table 4) for detecting papillomavirus infection and typing low-risk and high-risk oncogenic human papillomas by PCR.

Table 4. Kits produced by DNA-Technology for detecting human papillomavirus infection

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
HPV 16,18 PCR detection Kit	*	*	*	–	RU/IVD
HPV 16/18 multiplex	–	–	*	–	RUO
HPV 16, 31, 33, 35, 35H, 58, 52, 67	*	–	–	–	RUO
HPV 18, 45, 39, 59	*	–	–	–	RUO
HPV 51,26	*	–	–	–	RUO
HPV 6, 11	*	–	–	–	RUO
HPV 6/11 multiplex	–	–	*	–	RUO
HPV 6	–	–	*	–	RUO
HPV 11	–	–	*	–	RUO
HPV QUANT-4 (HPV 16,18)	–	–	*	*	RU/IVD
HPV QUANT-4 (HPV 6,11,16,18)	–	–	*	*	CE/IVD
HPV QUANT-15 (HPV 16, 31, 33, 35, 52, 58, 6, 11, 18, 39, 45, 59, 51, 56, 68)	–	–	*	*	CE/IVD
HPV QUANT-21 (HPV 6, 11, 44, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73,82)	–	–	*	*	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

Kit format:

- Strip tubes (8 pcs., 0.2 ml each) (only strip tubes for kit HPV QUANT);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C.

Shelf life:

- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months (except HPV QUANT, HPV 6/11 multiplex and HPV 16/18 multiplex kits – 6 months).

DNA extraction kits:

- *PREP-RAPID;*
- *PREP-NA;*
- *PREP-GS;*
- *PREP-NA-PLUS (for use with HPV QUANT);*
- *PREP-GS-PLUS (for use with HPV QUANT).*

Specimen for screening:

Scrapings from mucosa of the urogenital tract.

Recommended additional reagents:

- SIC – sample intake control – 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 HPV QUANT, SIC is part of the kit.

Equipment required for analysis:

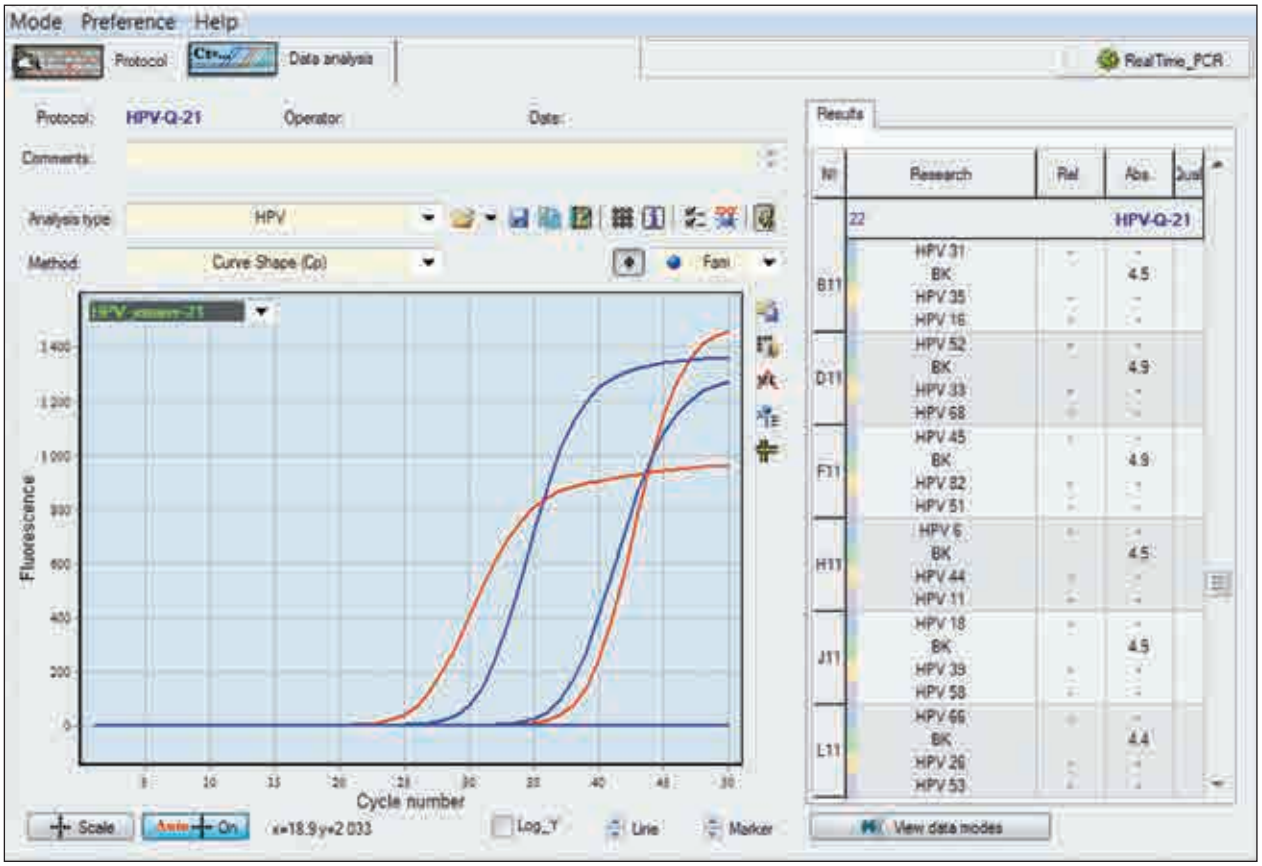
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories (except for HPV QUANT kits) and Rotor-Gene device produced by QIAGEN (only HPV 16,18)

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 3).

A



B

HPV

Date
Number of tube
Patient name
Sex
Age
Organization
Clinician name
Comments

Information about laboratory

Sample ID: 22

№	Name of research	Results		
		Relative, (X/SIC)	Quantitative, Lg (coples/sample)	Qualitative
1	HPV 31	not discovered	not discovered	
2	HPV 35	not discovered	not discovered	
3	HPV 16	not discovered	not discovered	
4	HPV 52	not discovered	not discovered	
5	HPV 33	not discovered	not discovered	
6	HPV 68	not discovered	not discovered	
7	HPV 45	not discovered	not discovered	
8	HPV 82	not discovered	not discovered	
9	HPV 51	not discovered	not discovered	
10	HPV 6	not discovered	not discovered	
11	HPV 44	not discovered	not discovered	
12	HPV 11	not discovered	not discovered	
13	HPV 18	not discovered	not discovered	
14	HPV 39	not discovered	not discovered	
15	HPV 58	not discovered	not discovered	
16	HPV 66	not discovered	not discovered	
17	HPV 26	not discovered	not discovered	
18	HPV 53	not discovered	not discovered	
19	HPV 59	not discovered	not discovered	
20	HPV 56	not discovered	not discovered	
21	HPV 73	not discovered	not discovered	
22	SIC		4.8	

Study was carried out

Date
Signature

Fig. 3. Analysis results for Rt optical measurements (DT devices)
The HPV-QUANT-21® *quantitative PCR Kit*
A – Optical measurement analysis (Fam channel)
B – Analysis results

4. HCV, HBV AND HIV VIRUSES

During diagnosis of *blood-borne infections*, PCR technology has great importance especially in blood banking. In 1995 on the recommendation of the National Institute for Biological Standards and Control, UK (NIBSC) under WHO a working group SoGAT was established to exchange experience in introducing gene testing for the most transfusion-dangerous viruses – HIV, hepatitis B, C, A viruses, parvovirus, and others for blood banking and other areas.

The experience of applying gene test proved the efficiency of this method in detecting donors- virus carriers, especially in seronegative period. Since July 1999, gene test has become mandatory in the EU countries for all donated blood and plasma. Currently, PCR technology solves the following blood banking tasks:

- Ensuring infection safety during the period of "seronegative window";
- Providing information that no infectious agents were found in the blood or blood components with ambiguous results of enzyme multiplied immunoassay obtained;
- Identifying true virus carriers among seropositive individuals.

In laboratory diagnosis of viral hepatitis and HIV infection, there is a number of problems that PCR method can resolve, thereby enabling a doctor to make an early diagnosis and commence treatment.

- Discrepancies between PCR results and enzyme-linked immunosorbent assay (ELISA) results – positive PCR result and negative ELISA result – are the most urgent challenges in detecting hepatitis C and human immunodeficiency viruses. This fact can be explained by the period of "serological window". Typically detectable amounts of HIV antibodies appear in the blood 2-10 weeks after infecting. However, variability of periods can be quite large. For example, HIV antibodies are detected within three months after infecting in 90-95 % of those infected, after six months in 5-9 % of patients, and at a later date in 0.5-1 %. Detection of DNA/RNA virus by PCR method reduces the duration of the period of "serological window" by 11 days on average and helps detect the pathogen within 1-2 weeks after infecting.
- With PCR it is possible to detect the RNA of hepatitis C virus not only in blood serum but also in liver biopsy specimens, which is important in confirming the role of hepatitis C virus in the formation of hepatocellular carcinoma. In such patients, hepatitis C virus RNA is detected in hepatocytes and in the absence of anti-HCV and HCV RNA in blood serum. Anti-HCV does not appear at all among a number of patients with self-limited course of infection.
- Occurrence of maternal antibodies in infants born by infected mothers can also distort the true picture. Since maternal IgG antibodies penetrate through the blood-placenta barrier, the antibody test result in babies born by such mothers will be positive for a long time (up to a year and a half after the birth). In this case, the presence or absence of the virus in the body of a baby can only be proven by using direct methods for detecting the virus, PCR being one of them.

Detecting the viral genotype is an important aspect that determines treatment management and further care of hepatitis C patients. This is explained by the fact that HCV is characterized by high variability and the presence of several variants of genotype. In clinical practice, it is important to distinguish 5 HCV subtypes: 1a, 1b, 2a, 2b, 3a.

So, among patients with subtype 1b, chronization of HCV infection occurs in 90 % of cases, while for genotype 2a and 3A patients, it occurs in 33-50 %. Genotype 1b infection is accompanied by more severe disease, development of liver cirrhosis and hepatocellular carcinoma. Patients with subtype 3a have franker steatosis and biliary tract lesions, as well as higher ALT levels as compared with the patients having HCV genotype 1b. At the same time fibrosis levels are more pronounced in patients with subtype 1b virus.

Determining the viral loads is the key factor in viral hepatitis and HIV treatment:

- Timing of the first therapy – for HIV infection, prescription of HAART depends on CD4+ concentration and viral load.
- Monitoring of the efficacy of antiviral therapy. For treatment of viral hepatitis and HIV infection, a range of pharmaceutical products has been developed whose effectiveness can be different for each individual patient. When selecting an adequate dosage scheme, it is required to conduct quantitative analysis of the virus content in the blood (viral load) by real-time PCR as the primary measure of efficiency.

Standardization of quantitative methods for determining DNA in the blood serum resulted in use of *International Units* (IUs). IUs do not reflect the actual number of viral particles (DNA copies) in a blood plasma sample; depending on the test system used.

Presently, the results of studies of genetic polymorphisms of several cytokines are used to determine the sensitivity to treatment of chronic hepatitis C and possibility of spontaneous infection elimination as one of the key factors of the immune system. From this perspective, identification of *polymorphisms of interleukin 28 (IL 28B) gene* is crucial.

Taking into account the current tasks PCR-based analysis is effective in the activities of infection disease specialists, hepatologists, professionals in the area of HIV infection prevention and control.

DNA-Technology offers the following kits (see Table 5) for detection and quantitative analysis of viral hepatitis and HIV as well as HCV typing by PCR.

Table 5. Kits produced by DNA-Technology for detecting viral hepatitis and HIV

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Hepatitis A virus	*	*	–	–	RUO
Hepatitis B virus	*	*	*	*	RU/IVD
Hepatitis C virus	*	*	*	*	RU/IVD
Hepatitis D virus	*	*	–	–	RUO
Hepatitis G virus	*	–	–	–	RUO
Hepatitis C virus genotyping	–	–	*	–	RU/IVD
Human immunodeficiency virus	–	*	*	*	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);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C.

Shelf life:

- Forez – 9 months (except HBV kit – 12 months);
- FLASH – 9 months (except HBV kit – 12 months);
- Rt – 9 months (except HBV kit – 12 months)

Kits for DNA/RNA extraction:

- *PREP-NA* (for kits used to detect hepatitis B virus DNA, hepatitis C virus RNA, HIV RNA, and for HCV genotyping)
- *PREP-NA* is included in kits for quantitative analysis of hepatitis B virus DNA, hepatitis C virus RNA and Hepatitis C virus genotyping.

Specimen for screening:

- Blood plasma.

Equipment required for analysis:

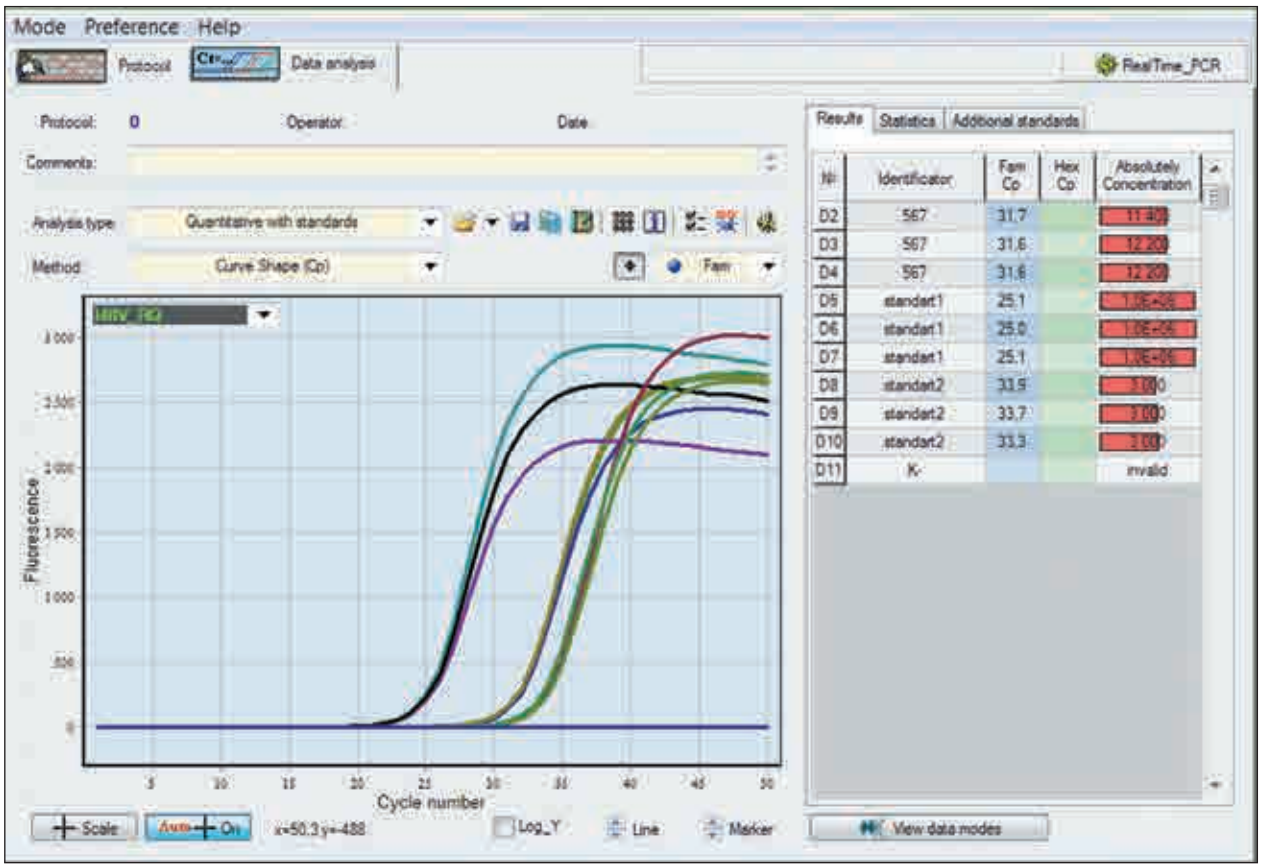
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories and Rotor-Gene Q devices produced by QIAGEN.

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (Fig. 4).

A



B Qualitative analysis

Number of the hole	Identificator of the tube	Cp, Fam	Cp, Hex	Result
D2	567 (HBV_RQ)	31.7		11 400
D3	567 (HBV_RQ)	31.6		12 200
D4	567 (HBV_RQ)	31.6		12 200
D5	standart1 (HBV_RQ)	25.1		1.0E+0.6
D6	standart1 (HBV_RQ)	25.0		1.0E+0.6
D7	standart1 (HBV_RQ)	25.1		1.0E+0.6
D8	standart2 (HBV_RQ)	33.9		3 000
D9	standart2 (HBV_RQ)	33.7		3 000
D10	standart2 (HBV_RQ)	33.3		3 000
D11	K– (HBV_RQ)			invalid

Fig. 4. Results of *Rt* qualitative analysis (DT devices) HBV REAL-TIME PCR Detection Kit
A – Optical measurement analysis (Fam channel)
B – Analysis results

5. RESPIRATORY TRACT INFECTIONS

According to WHO, respiratory tract infections are one of the main causes of sickness rate and mortality both at infancy and at older ages. About 70 % of these infections affect the upper respiratory tract and middle ear region.

Diagnosis and treatment of respiratory infections still remain the focus of attention among both therapists and pediatricians since these infections account for over 90 % of all outpatients' complaints, especially during autumn and winter seasons.

Corynebacterium diphtheriae

Corynebacterium is a genus of Gram-positive rod-shaped bacteria that causes diphtheria. Diphtheria is transmitted by air and is characterized by local fibrinous inflammation, especially that of the mucous membranes of the oropharynx and nasopharynx, as well as by general intoxication and visceral injury. Severe consequences of diphtheria are associated with release of toxins into blood and their fixation in tissues, which results in the nervous (parenchymatous neuritis) and cardiovascular (myocarditis) systems disorders.

The difficulties in diagnosing toxigenic strains of corynebacterium diphtheria are associated with the symptoms typical for catarrhal diseases as well as with occurrence of abnormal clinical forms and prolonged asymptomatic carriage of the infectious agent. PCR analysis of diphtheria make it possible to obtain results within 24 hours and determine toxigenicity of the strains of microorganisms, regardless of the beginning of antibiotic therapy.

Specimen for PCR testing:

- Throat swab;
- Tonsillar smear;
- Throat lavage.

Bordetella pertussis

Bordetella pertussis causes whooping cough (pertussis), an acute respiratory disease that is clinically manifested in the form of spasmodic coughing spells, infection of the respiratory, nervous and cardiovascular systems without primary intoxication and temperature reaction. Infected people and bacilli carriers can serve as the infection source. The infection is transmitted by air. It is more common among children under 5, in adults the clinical course is atypical.

The "golden standard" for laboratory diagnosis of pertussis implies inoculation of nasopharyngeal secretions during the first 2-3 weeks of illness; later the test validity declines. Applying serological tests (antibodies to *Bordetella pertussis* antigens and *Bordetella pertussis* toxin) is reasonable at the later stages of infection; the test has high specificity but relatively low sensitivity.

Community-acquired pneumonia is one of the primary causes of people's morbidity, hospitalization and mortality, especially among senior people and those with background diseases.

According to the Russian and foreign researchers, St.pneumoniae is a dominant etiological agent of pneumonia. Among other typical bacterial causative agents of pneumonia a significant etiological role belongs to K.pneumoniae and a group of micro-organisms (obligate and facultative intracellular parasites) resistant to β-lactam antibiotics: mycoplasma pneumonia, chlamidophila pneumonia and legionella pneumophila.

Specimen for PCR testing:

- Epithelial cell scrapings from the nasopharynx;
- Phlegm;
- Aspirate;
- Broncho-alveolar lavage;
- Blood.

Legionella pneumophila

Gram-negative bacterium proliferates in air conditioning systems, showerheads, humidifiers and inhalers and causes infectious disease legionellosis or Legionnaires ' disease. Sporadic cases are rare, as a rule outbreaks occur. Clinical implications are fever, weakness and discomfort, loss of appetite and dry cough. There are two forms of the disease: mild (flu-like) and severe (in the form of pneumonia). Chest X-ray reveals extensive lung damage.

Diagnosis is based on the results of bacteriological inoculation and serological tests (reaction to indirect immunofluorescence). PCR-based diagnostics are used to confirm the diagnosis.

Specimen for PCR testing:

- From patients: blood plasma, mucosal scrapings, broncho-alveolar lavage;
- From the environment

Chlamydophila (Chlamydia) pneumoniae

This is an obligate intracellular parasite. According to the statistics, *C. pneumoniae* is the causative agent of about 5-15 % of the community-acquired pneumonia cases. Prevalence of the disease caused by this infectious agent tends to increase. Mostly *C. pneumoniae* is clinically manifested in acute form – pneumonia, as well as prolonged bronchitis, pharyngitis, laryngitis, sinusitis and arthritis.

Considering the apparent growth of respiratory Chlamydia cases, it is strongly recommended to test at least patients with pneumonia, bronchial asthma and chronic obstructive pulmonary disease (COPD) for *C. pneumoniae*.

Based on the clinical presentation only, it is often impossible to make a clear diagnose. Therefore, special emphasis should be given to laboratory diagnostics to

allow for establishing the infection etiology and making diagnostics at early stages of the disease.

Laboratory diagnostics with using microbiological techniques is complicated. The comparison between sensitivity of microscopical method and PCR indicates that the frequency of pathogen detectability by microscopic examination is 10-12 % and by PCR at least 98 % respectively. With meeting the requirements for culture technique, the sensitivity for chlamydia diagnostics is 60-80 %, while PCR shows the sensitivity at least 95-98 %.

Specimen for PCR testing:

- Phlegm;
- Blood;
- Pleural fluid;
- Aspirates from sinus and middle ear;
- Endotracheal aspirates;
- Broncho-alveolar lavage;
- Biopsy specimens.

Mycoplasma pneumoniae

A single-celled Gram-negative microorganism without a cell wall. In terms of structural organization, mycoplasmas lie between bacteria and viruses. The fact that this microorganism can grow in cell-free media and can reproduce themselves excludes them from the virus family. They are regarded as hardly-cultivated microorganisms and considered to be superficial parasites of mucosa cells.

Among the factors that contribute to the development of a pathological process caused by *mycoplasma pneumoniae* there are a patient's age (younger than 5 years old and older than 50), smoking, hypothermia, chronic lung disease, chronic heart disease, chronic kidney disease, chronic gastrointestinal tract disease as well as impairment of overall non-specific human organism resistance (suppression of phagocytosis, bactericidin production, mucociliary clearance disorder, etc.).

The incubation period of the disease can last from 1 to 4 weeks. In most cases, it is only 12-14 days. The disease begins with respiratory syndrome (rhinopharyngitis and/or laryngotracheitis); otitis is possible too. Among extrapulmonary manifestations of mycoplasma pneumonia may be myalgia, sweating, gastrointestinal disorders and CNS disorders (headache and sleep disturbances).

The disease outcome is favorable, however, clinical symptoms and signs regress as well as that of radiographic signs proceeds slowly. At mycoplasma pneumonia a relapse of chronic diseases may occur.

Infection complications are manifested as respiratory distress syndrome, atelectasis, mediastinal adenopathy, pneumothorax, pleural effusion and pulmonary abscess. Extrapulmonary complications manifest themselves in the form of nervous system injury (meningoencephalitis, aseptic meningitis, encephalitis, ascending paralysis and transverse myelitis); haematological diseases (autoimmune hemolytic anemia, paroxysmal cold hemoglobinuria, Raynaud's phenomenon, disseminated intravascular coagulation, thrombocytopenia), cardiac complications (pericarditis, myocarditis, heart failure, complete AV block), skin and mucous membrane lesions (maculopapular rash and vesicular lesions; patients with rash may develop aphthae and conjunctivitis); articular manifestations (arthritis).

Differential diagnostics is performed in case of various pneumonias: viral, bacterial, fungal, pneumocystis, and also at tuberculosis.

Clinical, radiological and laboratory data regarding M.pneumoniae-caused infections are inadequate in order to make an accurate diagnosis based on them. Therefore laboratory diagnostics should include direct methods (first and foremost PCR) for detecting the microorganism in the biological material.

Specimen for PCR testing:

- Phlegm;
- Blood;
- Pleural fluid;
- Aspirates from sinus and middle ear;
- Endotracheal aspirates;
- Broncho-alveolar lavage;
- Biopsy specimens.

Streptococcus pneumoniae

Streptococcus pneumoniae is a natural inhabitant of a human upper respiratory tract. Streptococci are Gram-positive facultative anaerobic microorganisms. Pneumococci are characterized by the presence of strong polysaccharide capsule, which act as protection, inhibiting opsonization and subsequent phagocytosis. There are at least 90 different capsular types of *S. pneumoniae*, but the majority (>90 %) of invasive diseases are caused by 23 serotypes that are included as compounds in currently used polysaccharide vaccine.

β-hemolytic streptococci (that rarely causes pneumonia with healthy adults, as a rule against the background of diabetes or other serious illnesses) and pyogenic streptococci (that often causes pneumonia among children and young people) penetrate into the lungs airborne.

Streptococcus pneumoniae is rarely seen in adults and considerably more often (20 % of cases) in children. In the focal pneumonia pattern with healthy (non-immunosuppressed) people the share of the infection can amount to 10 %. In general this microbe causes mostly otitis, tonsillitis and pharyngitis. In rarer cases, pneumococcus can cause other localized infections (endocarditis, septic arthritis, primary peritonitis, cellulitis, etc.).

Bacterial inoculation is recognized as the golden standard for detecting the microorganism. PCR is used to confirm diagnosis and detect the microorganism against the background of commenced antibiotic therapy.

Specimen for PCR testing:

- Phlegm;
- Blood;
- Pleural fluid;
- Aspirates from sinus and middle ear;
- Endotracheal aspirates;
- Broncho-alveolar lavage;
- Biopsy specimens.

DNA-Technology offers the following kits (see Table 6) for detecting bacterial respiratory tract infections by PCR.

Table 6. Kits produced by DNA-Technology for detecting bacterial respiratory tract infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Chlamydia pneumoniae	*	–	*	–	RUO
Bordetella pertussis	*	*	*	–	RUO
Corynebacterium diphtheriae	*	*	*	–	RUO
Legionella pneumophila	*	*	*	–	CE/IVD
Mycoplasma pneumoniae	–	–	*	–	RUO
Streptococcus pneumoniae	–	–	*	–	RUO
Chlamydia pneumoniae/ Mycoplasma pneumoniae	–	–	*	–	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

Kit format:
- Strip tubes (8 pcs., 0.2 ml each);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C.

Shelf life:
- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months.

DNA extraction kits:
- *PREP-RAPID;*
- *PREP-NA (for RNA extraction);*
- *PREP-GS.*

Specimen for screening:
- Vesicles fluid;
- Scrapings from mucosa;
- Phlegm;
- Cerebrospinal fluid;
- Bronchial aspirate;
- Biopsy specimen;
- Blood;
- Surface swabs.

Recommended additional reagents:
SIC-sample intake control- 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.

Equipment required for analysis:
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories and Rotor-Gene Q devices produced by QIAGEN.

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

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

Influenza (flu) *is an acute infectious disease of the respiratory tract caused by the influenza virus. It is an acute respiratory viral infection* (**ARVI**). In terms of frequency and number of flu incidences, ARVI ranks first in the world, accounting for 95 % of all infectious diseases. The death rate from this disease remains high: 2 million people in the world die each year from flu. In Russia, per every 100 thousand people there are 2.7 children's deaths from influenza and 80 cases of deaths among persons older than 65.

Influenza viruses belong to the *Ortomyxoviridae* family, which includes 3 genera: *influenza A virus, influenza B virus* and *influenza C virus*. The antigenic properties of internal virion proteins identify influenza virus as genera A, B or C. Further grouping is done according to subtypes of surface proteins hemagglutinin and neuraminidase.

Viruses containing three hemagglutinin subtypes (H1, H2, H3) and two neuraminidase subtypes (N1, N2) are of epidemic concern for people. Influenza viruses A and B contain hemagglutinin and neuraminidase as the main structural and antigenic components of viral particles that have hemagglutinating and neuraminidase activities. Influenza C virus has no neuraminidase. It has a hemagglutinin-esterase (penetrating) protein (HEF).

Influenza A virus

It is worldwide and causes seasonal epidemics among humans, outbreaks of animal disease (H1N1 swine flu) and bird disease (H5N1 (bird flu). It regularly causes pandemics. The features of influenza type A virus genome are associated with a high degree of variability (point mutations, antigenic shift) that causes a variety of subtypes.

Influenza A viruses (H1N1 and H3N2 subtypes) that cause seasonal epidemics usually circulate among humans. There are also cases of human infection with influenza A viruses of other subtypes that are more characteristic for birds. Some of these subtypes (for example, H5N1) have pandemic potential.

Influenza B virus

When infected with influenza B virus the disease usually proceeds in a milder form, affecting mostly children and young adults. A characteristic feature of influenza B viruses is that it circulates only in human environment.

DNA-Technology offers the following kits (see Table 7) for detecting viral respiratory tract infections by PCR.

Table 7. Kits produced by DNA-Technology for detecting viral respiratory tract infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Influenza A virus (subtype H5N1)	–	*	*	–	RUO
Influenza A virus (subtype H1N1)	–	–	*	–	CE/IVD
Influenza A virus	–	–	*	–	CE/IVD
Influenza B virus	–	–	*	–	CE/IVD
Influenza A&B virus	–	–	*	–	CE/IVD
Influenza A virus, Influenza B virus Multiplex	–	–	*	–	RU/IVD
Acute viral respiratory infections Multiplex	–	–	*	–	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);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: from +2 to +8 °C (Taq-polymerase and reverse transcriptase at –20 °C).

Shelf life:
- FLASH – 12 months;
- Rt – 9 months (except influenza A virus kit (subtype H5N1) – 12 months)

Kits for RNA extraction:
- *PREP-NA;*
- *PREP-GS* (for detecting influenza B virus)

Specimen for PCR study:
- Human biomaterial (smears and swabs from the nasal cavity and oropharyngeal cavity);
- Material from sick and dead animals (smears and swabs from the trachea, nasal cavity, throat, cloacae; feces, internal organs).

Equipment required for analysis:
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories – only for influenza A virus (H5N1 subtype (bird flu)).

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for DT devices produced by DNA-Technology) (fig.5).

A

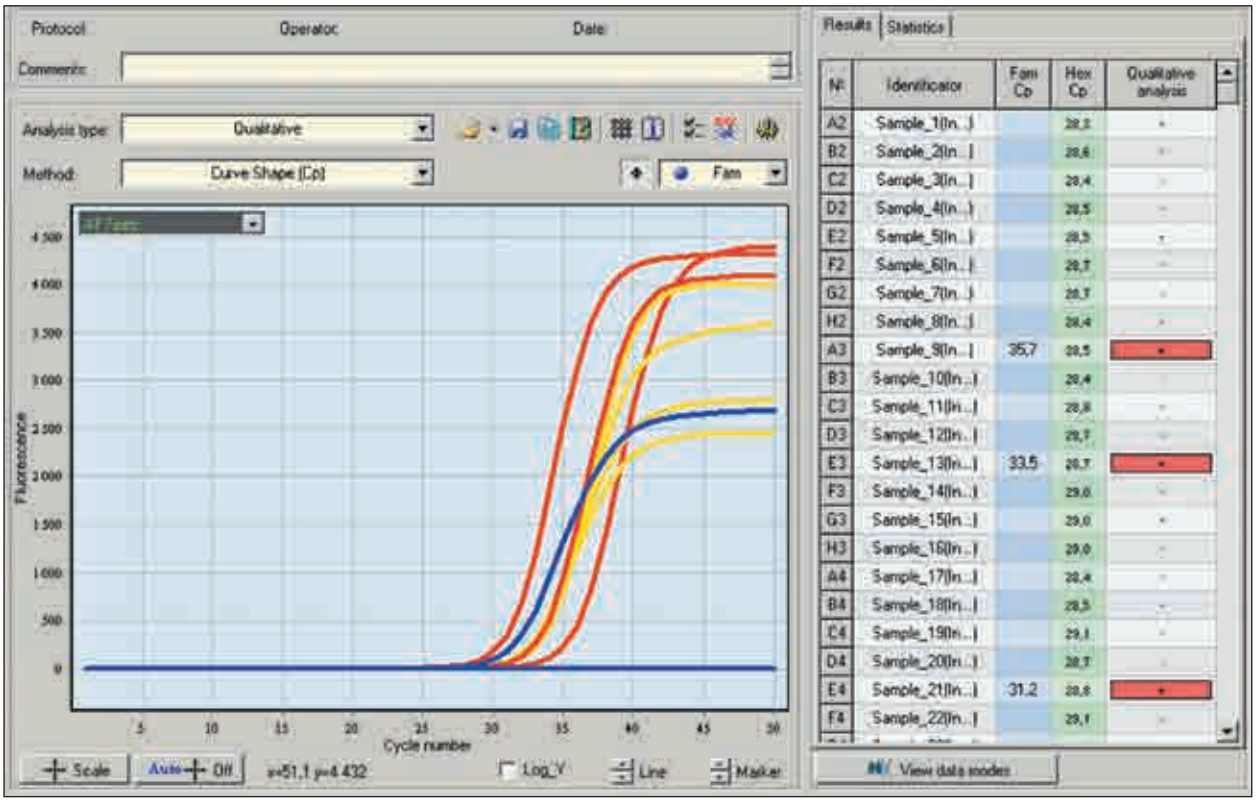


Fig. 5. Analysis results for Rt optical measurements (DT devices)
Influenza B virus Real time PCR Kit
A – Optical measurement analysis (Fam channel)

6. ESPECIALLY DANGEROUS AND NATURAL FOCAL INFECTIONS

Especially dangerous infections (EDIs). According to the International Health Regulations (IHR), EDIs are "infectious diseases that are included in the list of events which may constitute a public health emergency of international concern".

Annex 2 of IHR-2005 defines a list of such infections, divided into two groups. The first consists of "diseases, which are unusual or unexpected and may have serious public health impact": smallpox, poliomyelitis due to wild-type poliovirus, human influenza caused by a new subtype and severe acute respiratory syndrome (SARS).

The second group is defined as "diseases that have demonstrated the ability to cause serious public health impact and to spread rapidly internationally": cholera, pneumonic plague, yellow fever, viral hemorrhagic fevers and West Nile fever.

Natural feral herd infections are a special group of diseases that have evolutional lesions. Natural focus implies a biotope on the territory of a particular geographical landscape inhabited by animals whose species or interspecies differences support the pathogen circulation due to its transmission from one animal to another through blood-sucking arthropod vectors.

At the moment, an adverse epidemic situation for infections that were previously endemic in nature is forming. That is why, in order to take antiepidemic measures timely it is vital to apply quick-tests that can have the following characteristics:

- Obtaining the test results in the shortest possible time;
- Ability to carry out and complete analysis without isolating the required microorganism in a pure culture using native materials only;
- High specificity and sensitivity as a prerequisite for appropriate reliability of analysis;
- High efficiency, simplicity, availability and reproducibility of the analyses.

Borrelia burgdorferi

This is a microbial species belonging to the Spirochaetaceae family. The species contains 36 different types. 12 of these Borrelia species cause various diseases in humans – borrelioses (Lyme disease, relapsing fever) mainly of zooantroponoze nature with transmissible way of transferring the pathogen.

In Lyme disease, the major etiologic factors are *Borrelia burgdorferi*, *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana* species. Relapsing fever is often accompanied by bacteremia. Its pathogen – Borrelia recurrentis, which enters a human body through a bite of an insect infected.

The main transmission vectors are:

- Ticks – ixodic ticks (*Ixodes scapularis*), argasidae ticks (*Ornithodoros papillares* (village tick) and *Argas persicus* (Persian tick));
- Head lice, body lice and pubic lice.

Complex inflammatory and allergic skin changes, manifested in the form of specific erythema that is characteristic of Lyme disease usually develop at the site of tick bites.

The disease can begin with ill health, itching, swelling and redness at the site of a tick bite. As the disease progresses (or immediately in patients without local phase), borrelia spreads through the blood and lymph from the bite site to the internal organs. The infection symptoms develop within 1-1.5 months.

Borrelia can remain in a human body for a long time, causing chronic and relapsing disease. Lyme disease diagnosis at the late stage is especially difficult because of the severity of clinical polymorphism.

Laboratory diagnostic methods are essential in establishing the diagnosis of latent, subclinical forms and at the later stages. With PCR analysis, early diagnosis is possible during the serological window period.

Specimen for PCR testing:

- Infected tissues and body fluids of the patient;
- Ticks.

Vibrio cholerae

Vibrio cholerae is a Gram-negative, facultative anaerobic motile bacterium of the genus *Vibrio*. There are more than 140 serogroups of *V. cholera* at present; they are divided into agglutinated and non-agglutinated with generic cholera serum O1 (*V. cholerae non O1*). "Classical" cholera is caused by Vibrio cholerae serogroup O1, which includes two biovars: classic (*Vibrio cholerae biovar cholerae*) and El Tor (*Vibrio cholerae biovar eltor*).

Cholera is an acute infectious disease related to highly dangerous infections. It is characterized by fecal-oral mechanism of infection, small intestinal lesion, watery diarrhea, vomiting, rapid loss of body fluids and electrolytes with development of various degrees of dehydration up to hypovolemic shock and death.

The incubation period lasts from several hours to 5 days, usually 24-48 hours. The severity of the disease varies from latent and subclinical forms to severe conditions with sharp dehydration and death within 24-48

hours. In this regard, very fast and highly sensitive method, such as PCR analysis, is required for diagnostics.

Specimen for PCR testing:

- Feces and/or vomit, water.

Bacillus anthracis

Bacillus anthracis is a Gram-positive, spore-forming bacterium. It is an etiologic agent of anthrax. *Bacillus anthracis* is especially dangerous infectious disease of farm and wild animals of all species, and, occasionally, of humans. The disease occurs at lightning speed, hyperacute, acute and subacute – in sheep and cattle; acute, subacute and anginal – in pigs; predominantly as anthrax – in humans. It is characterized by intoxication, serous-hemorrhagic inflammation of skin, lymph nodes and internal organs; it occurs in a skin or septic form (intestinal and pulmonary forms are found in animals).

Diagnosis is based on clinical, epidemiological and laboratory data. Laboratory diagnosis includes bacterioscopic and bacteriological methods. But for the purpose of early diagnosis, immunofluorescence and PCR methods are used for laboratory diagnosis.

Specimen for PCR testing:

- Fluid of vesicles and carbuncles;
- Phlegm;
- Blood;
- Feces;
- Vomit (septic form).

Yersinia pestis

Yersinia pestis is a Gram-negative bacterium from the Enterobacteriaceae family. It is the infectious agent of bubonic plague and can also cause pneumonia (pulmonary form of plague) and septic plague. All the three forms are responsible for high mortality rate during epidemics.

Plague is an acute natural focal infectious disease in the group of quarantine infections. It occurs with very severe general condition, fever, damage to the lymph node, lung and other internal organs, often with development of sepsis. The disease is characterized by high mortality rates and extremely high infectivity.

In natural foci, the sources and reservoirs of the infectious agent are rodents – groundhogs, gophers and sandworts, mouse-like rodents and rats (gray and black). The rarer ones are house mice, and also double-toothed rodents, cats and camels. Vectors of the infectious agent are flea of different types.

Specimen for PCR testing:

- Lymph node festering punctate;
- Phlegm and blood of the patient;
- Discharge from fistulas and ulcers.

Francisella tularensis

Francisella tularensis is a Gram-negative bacillus with polymorphism. It is a causative agent of tularemia – a zoonotic disease that has natural foci. It is characterized by intoxication, fever and lymph node damage. It occurs in 3 forms, namely, bubonic, pulmonary and generalized (spreads throughout the body) forms. Tularemia bacillus vectors are hares, rabbits, water rats and voles. Epizootic outbreak periodically occurs in the natural foci. The infection is transmitted to humans through contact or directly from animals, or through contaminated food and water. Seldom infecting occurs through aspiration (when threshing bread and handling grains and forage products) and via blood-sucking arthropods (horsefly, ticks, mosquitoes, and others).

Bacteriological diagnostic methods for human tularemia have additional significance and are not always effective due to the biological characteristics of the pathogen and characteristics of human infection (low concentration of the infectious agent in tissues and organs). A much more efficient diagnosis method is the PCR method, which can significantly reduce the time of analysis.

Specimen for PCR testing:

- Bubo punctate, conjunctival discharge, film with tonsil, phlegm and others (from humans);
- Biopsy specimen (from animals).

DNA-Technology offers the following kits (see Table 8) for detecting especially dangerous and natural focal infections by PCR.

Table 8. Kits produced by DNA-technology for detecting especially dangerous and natural focal infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Borrelia burgdorferi	–	*	*	–	RU/IVD
Vibrio cholerae	–	*	*	–	RUO
Bacillus anthracis	–	–	*	–	RUO
Yersinia pestis	–	*	*	–	RUO
Francisella tularensis	–	*	–	–	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

Kit format:
- Strip tubes (8 pcs., 0.2 ml each);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: +2 to +8 °C.

Shelf life:
- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months.

DNA extraction kits:
- *PREP-RAPID;*
- *PREP-NA;*
- *PREP-GS.*

Specimen for screening:
- Scrapings from mucosa;
- Faeces;
- Cerebrospinal fluid;
- Biopsy specimen;
- Blood;
- Vomit;
- Phlegm;
- Vesicles fluid.

Equipment required for analysis:
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler device produced by Bio-Rad Laboratories.

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (fig.6).

A

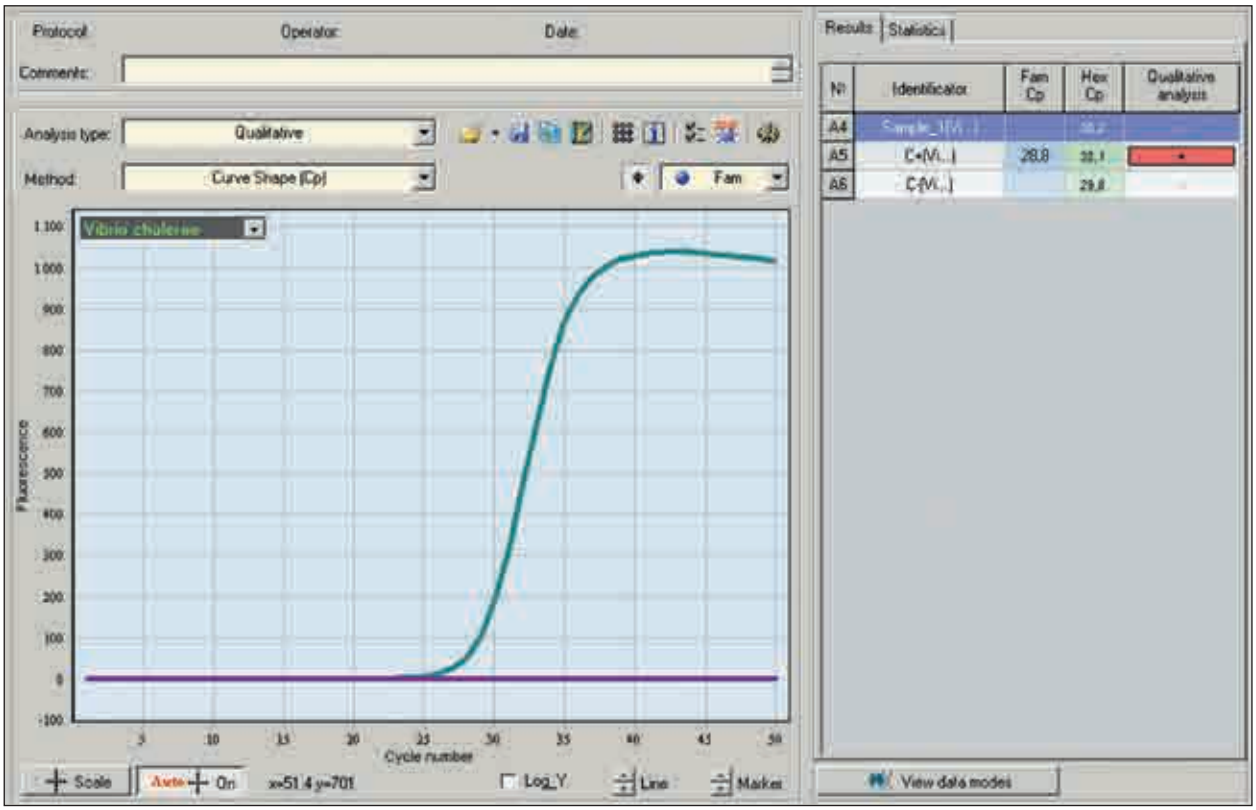


Fig. 6. Analysis results for Rt optical measurements (DT devices)
Vibrio cholera PCR Detection Kit
A – Optical measurement analysis (Fam channel)

7. OTHER INFECTIONS

MRS/MRSA Multiplex PCR Detection kit

The kit for detection of the DNA of *Staphylococcus spp.*, *Staphylococcus aureus* and *mecA* gene, which causes methicillin-resistance, by PCR in Real-time (MRS/MRSA).

In recent decades, the strains of pathogens that are resistant to the action of various drugs have become widespread worldwide. The resistance of microorganisms to the antibiotics can be natural or acquired, but forming the resistance in both cases is based on genetic factors.

Infectious disease caused by strains, which are resistant to antibiotics, are characterized characterized by longer duration, often require hospitalization with long stay in the hospital.

There are widely spread and well known methicillin-resistant strains of *Staphylococcus spp.* (**MRS** – methicillin-resistant *Staphylococcus*), including *Staphylococcus aureus* (**MRSA** – methicillin-resistant *Staphylococcus aureus*), coagulase-negative *Staphylococcuses: S. epidermidis, S. saprophyticus* and others (**MRCoNS** – methicillin-resistant coagulase-negative staphylococci).

Resistance to methicillin and oxacillin is caused by presence of penicillin binding protein (*PBP2A – penicillin binding protein 2A*) in the microorganism, which is coded by the *mecA* gene, transmitted as part of the mobile element ssc (*staphylococcal cassette chromosome*).

S. aureus – Gram-positive Staphylococcus spp, has a golden color due to the carotenoid pigments. In approximately 25-50 % of people asymptomatic colonization of S.aureus is present on the skin and mucous. In 30-60 % of cases, it leads to the development of infection in the presence of certain factors and conditions. These are:

- reception of various medications (immuno-suppressants, hormones, antibiotics, etc.);
- the impact of adverse environmental factors;
- decreased immunity;
- hospital treatment;
- pregnancy and childbirth;
- old age and the child age;
- traumatic condition (mechanical damage to the integrity of the skin, burns, etc.);
- chronic diseases (diabetes, cancer, etc.).

S. aureus remains one of the most important pathogens of human infections, causing a wide range of community-acquired and nosocomial diseases, from skin and soft tissue lesions to pneumonia, endocarditis, sepsis and toxic shock syndrome.

Path of transmission:

- airborne;
- airborne dust;
- contact;
- food;
- an artificial (related to medical procedures).

The ubiquity of S. aureus, the active colonization of the human, affected individuals of all age groups, etiological significance for a variety of diseases, especially heavy – all this determines the importance of a correct choice of drugs for the treatment. β-lactam antibiotics (oxacillin and methicillin) are traditionally included in the starting antibiotic therapy. It is therefore important to quickly get to the appointment of treatment and be sure to take into account the sensitivity spectrum of S. aureus.

According to the results of epidemiological monitoring of MRSA, it was found that the prevalence of methicillin-resistant strains has been uneven and varies from 2.6 % to 60 % of all isolates of S. aureus strains. The risk of death increases nearly three-fold times among patients whose bacteremia is caused by MRSA, compared with patients infected with methicillin-sensitive strains of S. aureus [8].

Other methicillin-resistant *Staphylococcuses* (for example MRCoNS, and especially *S. epidermidis*) – are microorganisms often causing lesions of shunts, joint prostheses, urinary and venous catheters and other serious nosocomial infections such as sepsis and opportunistic disease in patients with reduced immunity.

Recent studies have revealed that from 75 % to 90 % isolates of *S. epidermidis* from different hospitals of Europe and USA are methicillin-resistant. It is proven, that the propagation of *mecA* gene can occur between different species of *Staphylococcuses*.

Antibiotic Resistance of MRS/MRSA complicates treatment of infections caused by these microorganisms, particularly in an intensive care unit, in intensive care, oncohematology, cardiac surgery and transplantation, in severe patients with complicated history and a number of risk factors.

Methods of identification MRS/MRSA strains can be based on phenotypic and genotypic characteristics. The traditional definition of sensitivity to antibiotics is conducted via disk diffusion method or the method of serial dilutions. Study duration is usually more than 24 hours. Detection of methicillin-resistant strains of MRS/MRSA by PCR in real time, is based on the detection of DNA of *mecA* gene of staphylococcus, increases the speed of analysis and sensitivity. Thus there is no need to investigate the living culture of staphylococci, which helps to prevent the spread and continuous circulation of microorganisms in diagnostic and treatment facilities.

Due to the fact that Staphylococcus aureus is etiologically significant pathogen in chronic inflammatory processes of various localization, the National Association of specialists in infection control related to health care (NASIRH), it is recommended to conduct a survey of the following categories of patients:

- dermatitis, stafilo dermatitis;
- purulent processes of the skin and subcutaneous tissue (including a newborn);
- burns;
- inflammatory diseases of upper respiratory tract and eyes;
- diseases of the upper respiratory tract (bronchitis, tracheitis) and prolonged pneumonia;
- with inflammatory diseases of the urinary system and genital organs;
- acute postpartum endometritis;
- a blood infection;
- on admission to hospital from other hospitals or the availability of information about the hospitalization within the previous six months.

In addition, studies of medical staff within the clinical examination should be conducted on the subject of carriage of Staphylococcus aureus, or when applying for work in the obstetric, burn, trauma, surgery and other departments. Also to assess contamination of common user items in the medical and children's institutions during conducting sanitary-epidemiological control.

For the timely organization of isolation-restrictive measures in the study of NASIRH is high-lighted that it is important to use rapid diagnostic screening methods, such as PCR.

The advantaged of DNA diagnostics by PCR method:

- high analytical sensitivity;
- high analytical specifity of test;
- short time of analysis.

The study is carried out both in qualitative and quantitative analysis, which has a number of advantages:

- each tube with PCR-Mix contains internal control (IC) for evaluation of PCR quality;
- simultaneous detection of microorganism DNA and determination whether is it methicillin-resistant or not (*mec A* gene);
- combination of positive results establish the presence in the sample of MRS, MRSA, MSS and MSSA;
- In case of presence of the DNA of microorganism, its quantity is determined and calculated in decimal logarithm of the concentration.

Specimen for screening:

- sputum, milk, urine, cerebrospinal fluid;
- epithelial cell scrapings from the posterior pharyngeal wall, urethra, cervix, the posterior wall of the vagina;
- smears and swabs from the nasal cavity and oropharynx;
- feces;
- aspirate from the trachea;
- scrapings and swabs from the wound surface.

Group B Streptococcus (*Streptococcus agalactiae*)

Group B Streptococcus (GBS) is a part of normal vaginal flora that can be found in 5-35 % of pregnant women. In obstetrics, GBS commonly is associated with infections of urinary tract, chorioamnionitis, puerperal endometritis, bacteriaemia at birth and in postpartum period, premature rupture of membranes and preterm birth. The risk of intranatal infection during vaginal delivery estimated at 50-60 %. With reference to The American Society for Disease Control (Preventing Early-Onset Group B Strep Disease (GBS), 2016) the risk of infection for fill-term infant is 1-2 %, for preterm infant – 15-20 % and at gestational age less than 28 weeks – 100 %. Moreover, GBS is considered the main cause of newborn sepsis in USA. Annual morbidity is – 0,28 cases/1000 live-birth infants.

Contributory causes for clinical forms of infections (Prevention of Perinatal Group B Streptococcal Disease: Revised Guidelines from CDC, 2010):

- Obstetric causes:
  - preterm birth;
  - low body mass, prematurity (10-15 excess incidence);
  - 12-18 hours period without amniotic fluid (critical period is 24 hours);
  - woman's temperature rise above 38 °C;
  - placenta and/or amniotic fluid infection (clinically apparent chorioamnionitis, intra-uterine infection).
- GBS in maternal urea during pregnancy (marker of massive colonization) – screening and treatment of asymptomatic bacteriuria, determined as presence of bacteria in culture at quantities exciding 10^5 CFU/ml.

Recommendations on GBS prophylaxis

Prophylaxis is recommended	Prophylaxis is not recommended
Birth of GBS-infected child in anamnesis	GBS colonization during previous pregnancy
GBS bacteriuria at any trimester of current pregnancy	GBS bacteriuria during previous pregnancy
GBS positive vaginal-rectal screening at III trimester of current pregnancy	Negative vaginal-rectal screening at III trimester of current pregnancy
Unknown GBS status (screening is not performed, result is unknown) and any of the following points: • Delivery at less than 37 weeks of gestational age • premature rupture of membranes ≥18 hours • Mother's body temperature ≥38 °C • Positive GBS tests in course of pregnancy	Elective caesarian section in women with intact embryonic vesicle

GBS diagnostics (in accordance with «Prevention of Perinatal Group B Streptococcal Disease: Revised Guidelines from CDC», 2010):

- At 35-37 weeks gestational age routinely by cultural method with selective medium;
- In case of onset of labor in women with unknown GBS status, the molecular genetic methods are recommended for intranatal research.

Samples for PCR assay:

- Vaginal scrape;
- Rectal scrape;
- Nasopharyngeal scrape;
- Urea;
- Cerebrospinal fluid;
- Autopsy tissues;
- Blood.

Toxoplasma gondii

Toxoplasma gondii is an intracellular, parasitic protozoan and a causative agent of toxoplasmosis. The disease is characterized by polymorphic clinical picture (lymphadenopathy, nervous system damage, enlarged liver and spleen, skeletal muscle damage and myocardial damage). It is chronic in nature.

Toxoplasmosis falls into the group of TORCH infections considered potentially harmful to a child's development and to the health of pregnant women. Congenital toxoplasmosis is the most severe; so special attention is given to diagnostics of the disease during pregnancy.

There is a potential risk of transplacental transmission (15 % in the first trimester and 65 % at the end of pregnancy).

Only the mother's primary infection results in congenital infection. As the result of the infection in the first trimester of pregnancy and development of congenital toxoplasmosis, the fetus either dies or the baby is born with symptoms of severe congenital toxoplasmosis: intoxication, fever, jaundice, liver infection, spleen infection, lymph node infection and CNS infection (encephalomyelitis). The classic triad of hydrocephalus, chorioretinitis and intracranial calcifications are found in infants infected during the first trimester of pregnancy. Newborns infected in late pregnancy are usually born without any clinical signs of the disease.

Among people with normal immunity, toxoplasmosis usually occurs without clinical manifestations (in latent form). When the immune system is suppressed, the disease may occur in acute (most severe), subacute or chronic (lightest) forms. The chronic form and carrier state can turn into acute form among patients with immunosuppression caused by HIV infection, organ transplantation, chemotherapy, cancer and other diseases, as well as in patients under stress and pregnant women. Among patients with immune deficiencies, including AIDS, the disease becomes severe, mostly fatal in nature.

Laboratory diagnosis of toxoplasmosis is performed using ELISA, while PCR is used to confirm the diagnosis.

Specimen for PCR testing:

- Scrapings/ dab from the endocervical canal, cervix uteri and vaginal vault;
- Blood;
- Cerebrospinal fluid;
- Lymph node punctates, tonsils;
- Remnants of fetal membranes;
- Biopsy specimens of affected organs.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive aerobic microorganism. It is a human pathogen. The disease source: rodents, herbivores, birds releasing listeria into the environment. Listeriosis is a contagious zoonotic disease. Its incubation period lasts from 2 to 4 weeks. In asymptomatic chronic or acute nature, it affects various organs and systems. The main forms of the disease are anginal-septic and nervous forms.

When in a body, listeria multiplies in the regional lymph nodes and then enters the bloodstream. This results in development of bacteremia. The clinical picture of the disease is varied. The acute form begins with a temperature rise up to 38-40 °C, intoxication, sore throat, enlarged tonsils, tonsillitis symptoms and enlarged cervical lymph nodes. The clinical picture of the disease depends on the involvement of a particular organ in the pathological process. There are possible slow chronic pelvic inflammatory diseases. Colpitis and endotservit can often be the only manifestations of listeriosis.

Among pregnant women, listeriosis resembles an acute viral infection or quinsy. A fetus may die if it is infected at the later stages of pregnancy. In newborns, the infection occurs in the form of sepsis and meningitis; mortality can reach 50 %.

Clinical diagnosis of the pathogen is complicated. It is conducted using bacteriological and serological techniques. Currently, PCR method is used to confirm the diagnosis.

Specimen for PCR testing:

- Blood plasma (for septic form);
- Cerebrospinal fluid (for nervous form);
- Lymph node punctate;
- Tonsillar smears;
- Throat swab;
- Eye discharge;
- Amniotic fluid;
- Vaginal discharge;
- Smears/scrapings from the cervix and cervical canal;
- For newborns: meconium, blood, urine, cerebrospinal fluid.

Helicobacter pylori

Helicobacter pylori is a spiral-shaped Gram-negative bacteria found in patients with peptic ulcer (peptic ulcer disease), gastritis, non-ulcer dyspepsia and stomach cancer.

Laboratory diagnostics is aimed at diagnosis and follow-up. All the methods currently used can be divided into direct and indirect, non-invasive and invasive.

PCR method is a direct non-invasive method of diagnosis, which can detect the presence of *H. pylori* both in vegetative and in coccal form.

Specimen for PCR testing:

- Biopsy specimen;
- Saliva;
- Faeces.

The biomaterial should be collected taking into account localization and staging of the clinical process.

Streptococcus pyogenes

Streptococcus pyogenes is a Gram-negative, non-motile, non-spore-forming microbe with severe hemolysis. It occurs everywhere and often colonizes human skin and mucous membranes. The main modes of transmission are by air, contact and food.

It causes such infections as acute tonsillopharyngitis, acute otitis media, peritonsillar abscess, skin and soft tissue infections (pyoderma, impetigo, erysipelas, and scarlet fever), pneumonia and puerperal sepsis. Invasive infection manifests itself in the form of necrotizing fasciitis associated with streptococcal toxic shock syndrome.

The complications include acute rheumatic fever with secondary lesion of the mitral or aortic valves of the heart and acute glomerulonephritis.

Streptococcal infection is also associated with emergence of neurological obsessive-compulsive disorders in children.

About 20 % of pregnant women are bacilli carriers of the pathogen (nasopharynx, vagina and perianal region). A pregnant woman may experience tonsillitis, pharyngitis, pyoderma, urinary infection, horionamnionit, endometritis and puerperal sepsis. Infection is transferred to the child at birth with subsequent risk of neonatal sepsis,

especially during prolonged low-amniotic fluid interval.

The purpose of laboratory diagnosis is to detect the infectious agent within shortest time possible. This is when the use of PCR method is of advantage.

Specimen for PCR testing:

- Throat swab;
- Phlegm;
- Blood;
- Biopsy specimens;
- Synovial fluid;
- Wound fluid;
- Cerebrospinal fluid;
- Urine.

Mycobacterium tuberculosis

At the moment, there is a significant increase in incidence of tuberculosis in the world: every year there are nearly 8 million new TB cases. About 3 million people die from the disease every year.

Currently, initial laboratory analysis of specimen to detect the causative agent is done in general medical institutions through direct microscopic examinations. The most informative culture examination is carried out only in bacteriological laboratories of TB facilities at regional and major centers.

Nevertheless, routine diagnostics and typing of mycobacterium tuberculosis by microbiological methods take from 3 weeks to 3 months, which could adversely affect the effectiveness of therapy. Moreover, the nature of the disease has changed: every year, there is an increase in cases of primary tuberculosis caused by strains resistant to one or more antimycobacterial drugs. The usual antibiotic therapy will be ineffective in treating such patients because the infectious process is often severe and fast.

In this regard, there is an urgent need for detecting resistant strains.

The use of real-time PCR to detect results will allow for highly sensitive analysis both in a specialized facility and at a general medical institution at the initial stage.

Specimen for PCR testing:

- Blood;
- Phlegm;
- Urine;
- Pleural fluid;
- Biopsy specimens.

Human Parvovirus B19

From the family of human parvoviruses, only parvovirus B19 is pathogenic. It is a causative agent of infectious erythema, which also causes aplastic crisis in hereditary microspherocytosis and hemoglobinopathies, and erythroid aplasia in immunodeficiencies.

It is established that the frequency of B19 virus infection among pregnant women is one case per every 400 pregnancies. Moreover, it is shown that for the majority of pregnant women, the infection is asymptomatic in nature and is not the cause for pregnancy termination as the virus is not teratogenic in nature. However, the virus can cause serious pregnancy complications, especially during the period between 20 and 28 weeks of prenatal development. Fetal death occurs in 10% of pregnant women. Active infection leads to fetal anemia, which is the major factor in the development of hydrops and ascites. If appropriate treatment is not administered, parvovirus infection can lead to fetal death in the II trimester or to stillbirth.

Specimen for PCR testing:

- Samples of amniotic fluid;
- Exudate from the peritoneal and pleural cavities, which are obtained during prenatal diagnosis;
- Blood;
- Amniotic fluid.

DNA-Technology offers the following kits (see Table 9) for detecting the above-mentioned infections by PCR method.

Table 9. Kits produced by DNA-Technology for detecting other infections

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Streptococcus pyogenes	*	*	*	–	RUO
MRS/MRSA	–	–	*	–	RUO
Streptococcus agalactiae	*	*	*	–	RUO
Toxoplasma gondii	*	–	*	–	RUO
Human Parvovirus B19	–	–	*	–	RUO
Listeria monocytogenes	–	*	*	–	RU/IVD
Helicobacter pylori	*	*	*	–	RUO
Mycobacterium complex (Mycobacterium tuberculosis/ Mycobacterium bovis)	*	*	*	–	CE/IVD
MRS/MRSA Multiplex	–	–	*	–	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

Kit format:
- Strip tubes (8 pcs., 0.2 ml each);
- Single tubes (0.5 ml or 0.2 ml).

Storage temperature: +2 to +8 °C.

Shelf life:
- Forez – 9 months;
- FLASH – 12 months;
- Rt – 12 months (except kits for detecting MRS/MRSA – 6 months).

DNA extraction kits:
- *PREP-RAPID;*
- *PREP-NA;*
- *PREP-GS.*

Specimen for screening:
- Scrapings from mucosa;
- Faeces;
- Cerebrospinal fluid;
- Biopsy specimen;
- Blood.

Equipment required for analysis:
- For *FLASH* kits: Gene, Gene-4 or counterparts
- For *Rt* kits:
  - DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
  - IQ5 Cycler devices produced by Bio-Rad Laboratories and Rotor-Gene devices produced by QIAGEN

The following additional equipment is needed for analysis using strip tubes:
Strip plastic rack and centrifuge (vortex) rotor.

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

III. PCR IN THE STUDY OF MICROBIOME COMPOSITION

III. PCR IN THE STUDY OF MICROBIOME COMPOSITION

8. FEMOFLOR®

8.1. The FEMOFLOR® Real-Time PCR Kits

Vaginal microbiome composition is a complex dynamic system, which at any given time is affected by many endogenous and exogenous factors, including age, sexual behavior and obstetric history, hormonal status and hormonal disorders, use of hormone replacement therapy and combined oral contraceptives, antibiotic therapy and presence of extragenital pathology.

The status of vaginal microbiome composition has a serious impact on maintenance of the reproductive function and as a consequence, on quality of women's life.

Diseases caused by opportunistic microflora may occur both with clinical and asymptomatic manifestations. The asymptomatic course of the disease often leads patients to late visiting a doctor, thereby allowing for serious complications. It was found that diseases caused by opportunistic pathogens increase the risk of, sexually transmitted diseases and HIV infection. Non-promptly diagnosed infections associated with opportunistic pathogenic microflora can cause female reproductive disorders, spontaneous abortion, premature birth, intrauterine infection, low fetal weight, postnatal complications and pelvic complications after surgery.

FEMOFLOR® Real-Time PCR Kit allows to perform quantitative analysis of the total bacterial mass, urogenital, normal microflora (lactobacilli typical for female urogenital tract) and complex of aerobic and anaerobic microorganisms, mycoplasmas and Candida fungi that are involved in development of dysbiotic processes in urogenital microbiome composition from one bioassay test (see Table 10).

Table 10. The FEMOFLOR® Real-Time PCR Kit

GROUP	Specific components	Femoflor®-16
Control	Positive control	•
	Sample intake control	•
TBM	Total bacterial mass	•
	Lactobacillus spp.*	•
Aerobic microorganism (facultative anaerobes)	*Enterobacteriaceae*	•
	Streptococcus spp.	•
	Staphylococcus spp.	•
Anaerobic microorganism (obligate anaerobes)	*Gardnerella vaginalis/ Prevotella bivia/ Porphyromonas spp.*	•
	Eubacterium spp.	•
	Sneathia spp./ Leptotrihia spp./ Fusobacterium spp.	•
	Megasphaera spp./ Veilonella spp./ Dialister spp.	•
	Lachnobacterium spp./ Clostridium spp.	•
	Mobiluncus spp./ Corynebacterium spp.	•
	Peptostreptococcus spp.	•
	Atopobium vaginae	•
Mycoplasma group	*Mycoplasma hominis, Mycoplasma genitalium*	•
	Ureaplasma (urealyticum + parvum)	•
Fungi	*Candida spp./Sample intake control*	•

**spp* – a broad group of micro-organisms, which belongs to this genus, but may not completely match the genus in its systematic sense.

The scope of the method:

- Comparison of the amount of lactobacillus with the total amount of bacteria allows **evaluating the evidence of disorders in microbiota**;
- Comparison of the amount of opportunistic biota with the amount of lactobacillus allows **determining etiologic significance** of various microorganisms in developing dysbiosis as well as the degree of its manifestation;
- Quantitative analysis of genomic DNA of human epithelial cells in bioassay ensures **quality control at the pre-analytical stage** and prevents from obtaining false-negative results of the analysis;
- Detection of etiologically significant opportunistic microorganisms and determination of the degree of dysbiosis manifestation allows a clinician to determine the scope of therapeutic maneuver and prescribe causal treatment;
- **An individual approach to therapy** permits to reduce a number of side effects caused by excess medication;
- For the first time it has become possible **to monitor drug-induced effects on the urogenital tract microbiota**, evaluate treatment efficacy and microbiota recovery.

In order to have the objective picture of urogenital tract microbiome composition the following factors should be considered:

- The clinical situations when this medical technology cannot be applied:
  - Earlier than 24-48 hours after colposcopy;
  - Earlier than 24 hours after ultrasound examination using a vaginal sensor;
  - Earlier than 2 weeks after taking drugs (probiotics eubiotics) containing microorganisms.
- The clinical situations when the use of this medical technology is limited:
  - Menstrual bleeding;
  - Earlier than 10 days after taking antimicrobials or local antiseptics;
  - Use of gonadotropin-releasing hormones;
  - Surgical castration;
  - Lactational amenorrhea.
- The clinical situations when the use of this medical technology is impossible for other purposes:
  - Clinically significant amounts of microorganisms in the biomaterial are less than the level of negative control for the respective microorganism;
  - There are substances in the analyzed sample that affect the ability or effectiveness of nucleic acids isolation from biomaterial and/or amplification reaction (ultrasound coupling gel, heparin, etc.).

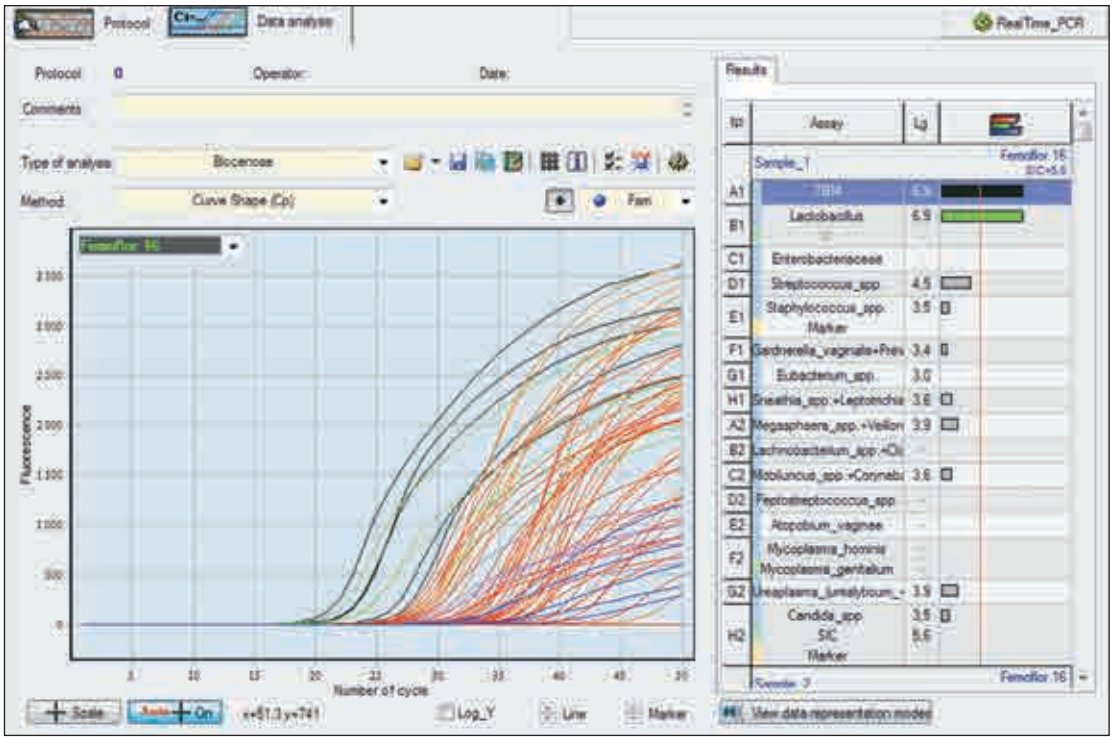
Based on the tasks set, the Femoflor-16 Real-Time PCR kit has seen developed for diagnosis of dysbiosis and treatment control (see Table 11).

Table 11. The FEMOFLOR®-16 Real-Time PCR Kit

№ of vial in a strip	Dye label/detection channel			Color of buffer	Color of paraffin
	Fam	Hex	Rox		
	Strip-1				
1	Total bacterial mass	–	–	Blue	White
2	*Lactobacillus spp.**	IC	–	Colorless	
3	*Enterobacterium spp.*	–	–		
4	*Streptococcus spp.*	–	–		
5	*Staphylococcus spp.*	–	Marker		
6	*Gardnerella vaginalis / Prevotella bivia / Porphyromonas spp.*	–	–		
7	*Eubacterium spp.*	–	–		
8	*Sneathia spp. / Leptotrihia spp. / Fusobacterium spp.*	–	–		
	Strip-2				
1	*Megasphaera spp./ Veilonella spp./ Dialister spp.*	–	–	Blue	Blue
2	*Lachnobacterium spp. / Clostridium spp.*	–	–	Colorless	
3	*Mobiluncus spp. / Corynebacterium spp.*	–	–		
4	*Peptostreptococcus spp.*	–	–		
5	*Atopobium vaginae*	–	–		
6	*Mycoplasma hominis*	*Mycoplasma genitalium*	–		
7	*Ureaplasma (urealyticum + parvum)*	–	–		
8	*Candida spp.*	SIC	Marker		

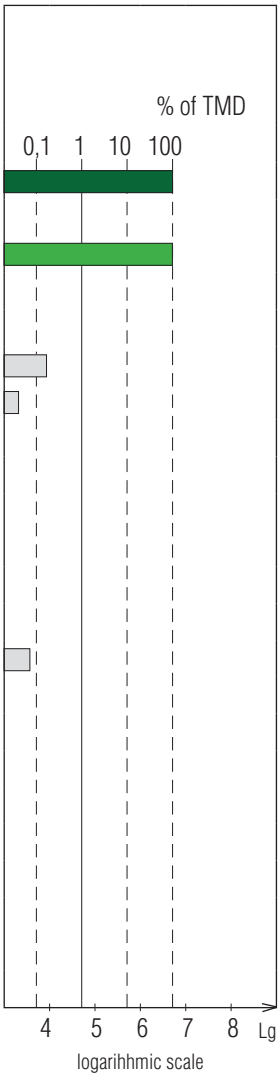
The software allows for processing the results automatically, preparing a clearly arranged laboratory report, showing the state of microbiota (Fig. 7).

A



B Sample ID: Sample_1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TMD)
	Control of sample intake	$10^{4,6}$ ■	
1	Total Bacterial Mass	$10^{6,6}$ □	
	Normal microflora		
2	Lactobacillus	$10^{6,6}$	0,0 (85-100 %) ■
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	not detected	□
4	Streptococcus spp.	$10^{3,8}$	-2,8 (0,1-0,2 %) □
5	Staphylococcus spp.	$10^{3,2}$	-3,4 (< 0,1 %) □
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	not detected	□
7	Eubacterium spp.	not detected	□
8	Sneathia spp. + Leptotrichia spp. + Fusobacterium spp.	not detected	□
9	Megasphaera spp. + Veillonella spp. + Dialister spp.	not detected	□
10	Lachnobacterium spp. + Clostridium spp.	not detected	□
11	Mobiluncus spp. + Corynebacterium spp.	$10^{3,7}$	-3,1 (< 0,1 %) □
12	Peptostreptococcus spp.	not detected	□
13	Atopobium vaginae	not detected	□
	Yeasts-LIKe fungi		
14	Candida spp.*	not detected □	
	Mycoplasma		
15	Mycoplasma hominis*	not detected □	
16	Ureaplasma (urealyticum + parvum)*	not detected □	
	Pathogenic microorganisms		
17	Mycoplasm genitalium**	not detected □	



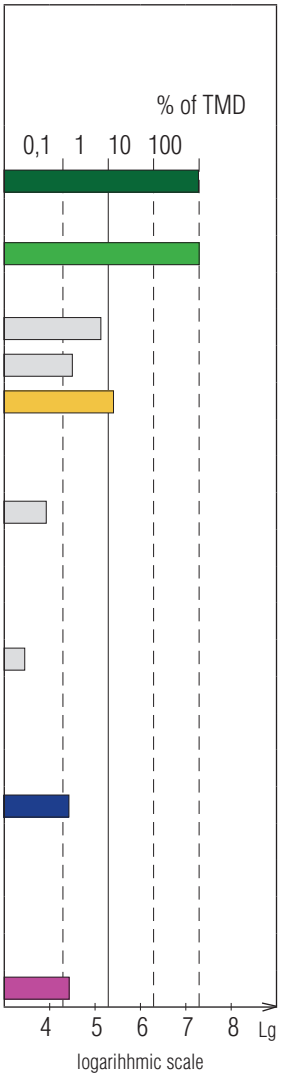
* Quantitative Analysis Lg(X)
** Quantitative Analysis

Conclusion:

Absolute normocenosis

C Sample ID: Sample_1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TMD)
	Control of sample intake	$10^{4,4}$ ■	
1	Total Bacterial Mass	$10^{7,3}$ □	
	Normal microflora		
2	Lactobacillus	$10^{7,3}$	0,0 (83-100 %) ■
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	$10^{5,0}$	-2,3 (0,4-0,6 %) □
4	Streptococcus spp.	$10^{4,4}$	-2,9 (0,1-0,1 %) □
5	Staphylococcus spp.	$10^{5,4}$	-1,9 (1,2-1,6 %) □
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	not detected	□
7	Eubacterium spp.	$10^{3,9}$	-3,4 (< 0,1 %) □
8	Sneathia spp. + Leptotrichia spp. + Fusobacterium spp.	not detected	□
9	Megasphaera spp. + Veillonella spp. + Dialister spp.	not detected	□
10	Lachnobacterium spp. + Clostridium spp.	not detected	□
11	Mobiluncus spp. + Corynebacterium spp.	$10^{3,7}$	-3,9 (< 0,1 %) □
12	Peptostreptococcus spp.	not detected	□
13	Atopobium vaginae	not detected	□
	Yeasts-LIKe fungi		
14	Candida spp.*	$10^{4,4}$ ■	
	Mycoplasma		
15	Mycoplasma hominis*	not detected □	
16	Ureaplasma (urealyticum + parvum)*	not detected □	
	Pathogenic microorganisms		
17	Mycoplasm genitalium**	detected ■	



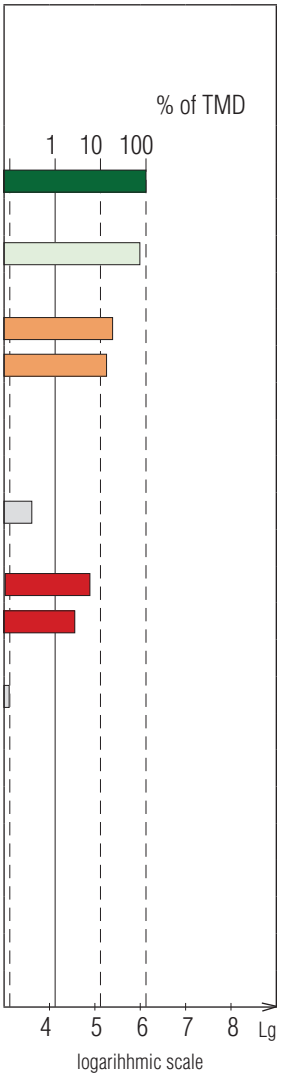
* Quantitative Analysis Lg(X)
** Quantitative Analysis

Conclusion:

Conventional normocenosis
Detected DNA of: Mycoplasma_genitalium

D

№	Name of research	Results	
		Quantitative	Relative Lg (O/TMD)
	Control of sample intake	$10^{4,6}$	
1	Total Bacterial Mass	$10^{6,1}$	
	Normal microflora		
2	Lactobacillus spp.	$10^{6,0}$	-0,2 (54-72 %)
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	$10^{5,4}$	-0,8 (14-19 %)
4	Streptococcus spp.	$10^{5,2}$	-1,0 (9-13 %)
5	Staphylococcus spp.	not detected	
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	not detected	
7	Eubacterium spp.	$10^{8,1}$	-2,5 (0,2-0,3 %)
8	Sneathia spp. + Leptotrichia spp. + Fusobacterium spp.	not detected	
9	Megasphaera spp. + Veillonella spp. + Dialister spp.	$10^{7,1}$	-1,3 (5-6 %)
10	Lachnobacterium spp. + Clostridium spp.	$10^{5,7}$	-1,4 (3-4 %)
11	Mobiluncus spp. + Corynebacterium spp.	not detected	
12	Peptostreptococcus spp.	$10^{3,1}$	-3,0 (< 0,1 %)
13	Atopobium vaginae	$10^{2,8}$	-3,3 (< 0,1 %)
	Yeasts-LIKe fungi		
14	Candida spp.*	not detected	
	Mycoplasma		
15	Mycoplasma hominis*	not detected	
16	Ureaplasma (urealyticum + parvum)*	not detected	
	Pathogenic microorganisms		
17	Mycoplasm genitalium**	not detected	



* Quantitative Analysis Lg(X)
** Quantitative Analysis

Conclusiion:

Moderate Anaerobic dysbiosis

Fig. 7. Analysis results for Rt optical measurements (DT devices)
A – Optical measurement analysis (Fam channel)
B-D – Form for presentation of results of the of the FEMOFLOR®-16 Real-Time PCR Kit

Laboratory report is prepared based on the algorithm used for analysis of the state of the microbiota of the urogenital tract of women of reproductive age (Fig. 8).

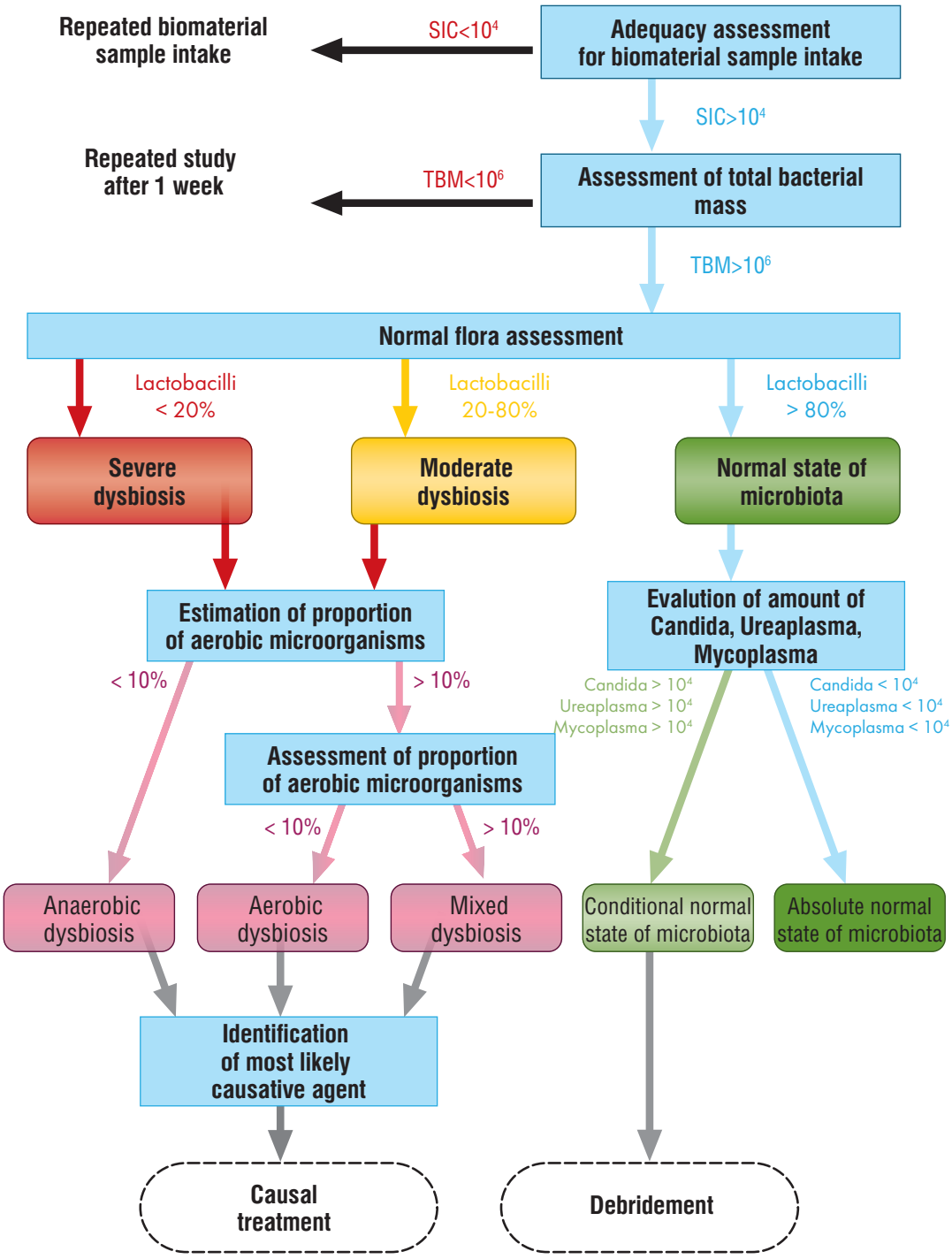


Fig. 8. Algorithm for urogenital microbiocenosis analysis for women of reproductive age

The main assessment parameters are:

- SIC (*Sample intake control* – human DNA). To obtain adequate results, SIC value should be greater than 10^4 GE/sample. In this case, bioassay can proceed with further analysis. If SIC is less than 10^4 GE/sample, the laboratory should contact a doctor for re-collection of biomaterial.
- Total bacterial mass (TBM) as an indicator of total bacterial load of biotope. TBM value should be in the range from 10^6 to 10^9 GE/sample (for women of reproductive age when analyzing scrapings from the posterolateral vaginal vault).
- **Amount of *Lactobacillus spp.* against TBM**. Based on this parameter, the following kinds are distinguished:
  - *Lactobacillus spp.* more than 80 % – NORMAL STATE OF MICROBIOTA (physiological vaginal microbiocenosis), characterized by dominance of normal flora;

- *Lactobacillus spp.* from 20 % to 80 % – MODERATE VAGINAL DYSBIOSIS;
- *Lactobacillus spp.* less than 20 % – SEVERE VAGINAL DYSBIOSIS.

- **presence and quantity of genital mycoplasmas, ureaplasma and yeasts** (in case if *Lactobacillus spp.* is over 80 % TBM):
  - If the concentration of the specified microorganisms is *less than 10^4 GE/sample* – ABSOLUTE NORMAL STATE OF MICROBIOTA;
  - If the concentration of the specified microorganisms is *more than 10^4 GE/sample* – Contingent NORMAL STATE OF MICROBIOTA (presence of *Mycoplasma genitalium* indicates the presence of pathogen and requires treatment with antibacterial drugs).
- In case of detecting vaginal dysbiosis (moderate or severe), it is necessary to determine its etiology by **quantitative ratio of opportunistic aerobic and anaerobic microorganisms**.
  - The amount of aerobic microorganisms which exceeds *10 %* out of total microorganisms detected (TMD) indicates AEROBIC vaginal dysbiosis;
  - The amount of anaerobic microorganisms which exceeds *10 %* indicates ANAEROBIC dysbiosis;
  - The amount of both groups of microorganisms which exceeds *10 %* out of total microorganisms detected (TMD) indicates MIXED dysbiosis.

DNA-Technology offers the following kits (see Table 12) for microbiota analysis of the female urogenital tract by PCR technique.

Table 12. The FEMOFLOR® Real-Time PCR Kit

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
FEMOFLOR®-16	–	–	–	*	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

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-NA-PLUS;*
- *PREP-GS-PLUS.*

Specimen for screening:
- Epithelial cell scrapings from the vagina (posterolateral vaginal wall), urethra and cervix.

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

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

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

8.2. The FEMOFLOR® SCREEN Real-Time PCR Detection Kit

DNA-Technology offers the FEMOFLOR® SCREEN Real-Time PCR Detection Kit for comprehensive etiologic diagnosis of urogenital infections during the initial consultation.

The FEMOFLOR® SCREEN Real-Time PCR Detection Kit allows to detect the presence or absence of a wide range of microorganisms in female urogenital tract from a **single** bioassay test, including the following (see Table 13):

- Basic (*Trichomonas vaginalis*);
- Bacteria (*Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium*);
- Viruses (differentiation of *Herpes simplex virus 1* and *Herpes simplex virus 2, Cytomegalovirus*).

Table 13. The FEMOFLOR® SCREEN Real-Time PCR Detection Kit

№ of the tube	Channel				Color of the buffer
	Fam	Hex	Rox	Rox	
1	Total bacterial mass	IC	–	–	Blue
2	Normoflora – *Lactobacillus spp.**	IC	–	–	Colorless
3	*Gardnerella vaginalis/ Prevotella bivia / Porphyromonas spp.*	IC	–	–	
4	*Ureaplasma (urealyticum + parvum)*	IC	–	–	
5	*Candida spp.*	SIC	Marker	–	
6	*Mycoplasma hominis*	IC	*Mycoplasma genitalium*	–	
7	*Trichomonas vaginalis*	IC	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	
8	*Herpes simplex virus 2*	IC	*Cytomegalovirus*	*Herpes simplex virus 1*	

FEMOFLOR® SCREEN provides quantitative analysis:

- Total bacterial mass for assessing bacterial load of the investigated biotope;
- *Lactobacillus* bacteria;
- Opportunistic microorganisms:
  - *Gardnerella vaginalis*
  - *Prevotella bivia /Porphyromonas spp.,*
  - *Ureaplasma urealyticum*
  - *Ureaplasma parvum,*
  - *Mycoplasma hominis,*
  - *Candida yeast.*

The FEMOFLOR® SCREEN Real-Time PCR Detection Kit includes sample intake control (SIC), which allows controlling the pre-analytical phase of the analysis and enables automatic results formation only when the amount of biomaterial is sufficient.

Indications for analysis:

- Subjective (complaints) and/or objective clinical symptoms of the urogenital tract of a patient;
- Preclinical examination;
- Assessment of therapy efficiency.

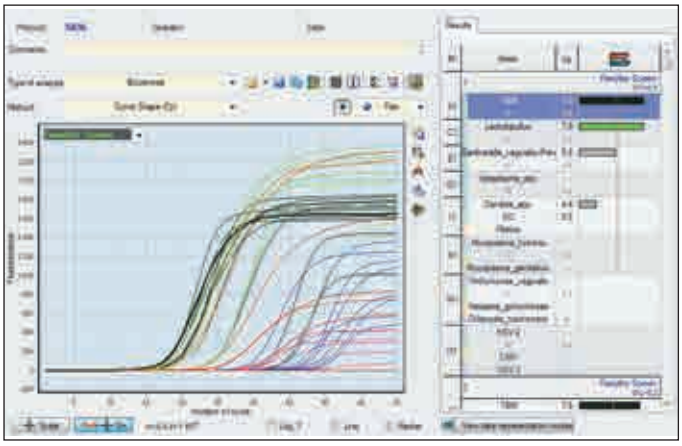
The FEMOFLOR® SCREEN enables to provide the following informaion during initial consultation :

- Etiological screening diagnostics of infectious process;
- 7 non-opportunistic pathogens (bacteria, viruses and protozoa);
- Diagnostics of dysbiotic disorders;
- Feasibility of determining the scope of treatment required;
- Possibility of carrying out dynamic observations;
- Monitoring the effectiveness of treatment;
- Monitoring the recovery of microbiota normal state;
- Quickly obtained results – feasibility to prescribe treatment promptly;
- Sampling quality control. Monitoring the adequacy of results obtained.

The FEMOFLOR® SCREEN is a modern diagnostics method:

- Control of pre-analytical phase (sampling control);
- Quick analysis (using DT devices, full cycle of analysis of samples (for DTprime) by 14 indicators each, takes 3 hours includig extraction)
- Software that facilitates the work of laboratory staff:
  - Screening report is issued by the software in a form that is suitable for a clinician;
  - A ready download file with the amplification program can be automatically downloaded to the device software, which significantly saves time and eliminates errors during the device manual programming (Fig. 9).

A



B

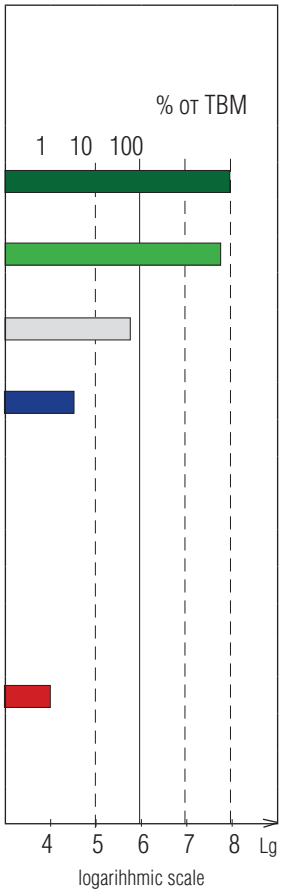
Study of urogenital tract biocenosis Femoflor Screen

Date
Number of tube
Patient name
Sex
Age
Organization
Clinician name
Comments

Information about laboratory

Sample ID: 1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Control of sample intake	$10^{5,5}$ ■	
1	Total Bacterial Mass	$10^{7,9}$ □	
	Normal microflora		
2	Lactobacillus spp.	$10^{7,9}$	-0,1 (70-94 %) ■
	Obligate anaerobic microorganisms		
3	Gardnerella vaginalis + Prevotella bivia + Porphyromonas spp.	$10^{5,8}$	-2,2 (0,6-0,8 %) □
	Yeasts-LIKe fungi		
4	Candida spp.*	$10^{4,4}$ ■	
	Mycoplasma		
5	Ureaplasma spp.*	not detected □	
6	Mycoplasma hominis*	not detected □	
	Pathogenic microorganisms		
7	Mycoplasm genitalium**	not detected □	
8	Trichomonas vaginalis**	not detected □	
9	Neisseria gonorrhoeae**	not detected □	
10	Chlamydia trachomatis**	detected ■	
11	HSV-2**	not detected □	
12	CMV**	not detected □	
13	HSV-1**	not detected □	



* Quantitative Analysis Lg(X)
** Quantitative Analysis

Fig. 9. Analysis results for Rt optical measurements (DT devices)
A – Optical measurement analysis (Fam channel)
B – Form for presentation of results of the of the
FEMOFLOR® SCREEN Real-Time PCR Detection Kit

DNA-Technology offers a kit (see Table 14) for screening of female urogenital genital tract microbiome composition by PCR method.

Table 14. The FEMOFLOR® SCREEN Real-Time PCR Detection Kit

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
FEMOFLOR® SCREEN	–	–	–	*	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-NA-PLUS;*
- *PREP-GS-PLUS.*

Specimen for screening:

- Epithelial cell scrapings from vagina (posterolateral vaults), urethra and cervix.

Equipment required for analysis:

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

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

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

9. THE ANDROFLOR® REAL-TIME PCR DETECTION KIT AND ANDROFLOR® SCREEN REAL-TIME PCR DETECTION KIT

Diseases of the male genital tract are the leading cause of male reproductive function impairment. This defines their social and economic significance, especially taking to account current decrease of birthrate.

The combined infection and inflammation process, which longevity and intensity defines the extent of fertility impairment, is considered the main reason of male genital tract diseases onset. Chronic inflammation provides prolonged toxic action on germinal epithelium, disrupts blood-testis barrier, affects rheological properties and chemical composition of the seminal fluid and can provoke development of autoimmune reactions like formation of antisperm antibodies.

The progression of inflammation provokes growth of activated immune cells level, which is accompanied with excessive production of oxygen free radicals and increased secretion of lymphokines and monkines promoting secondary inflammation in reproductive tissues.

According to Tenth Revision of International Classification of Diseases (ICD-10) the list of male genital tract diseases which can be caused by combined infection and inflammation include following diseases:

N34.1 Nonspecific urethritis
N34.2 Other urethritis
N34.3 Urethral syndrome, unspecified
N40 Enlarged prostate
N41.0 Acute prostatitis
N41.1 Chronic prostatitis
N45 Orchitis and epididymitis
N48.1 Balanitis
N48.6 Induration penis plastica
N49.0 Inflammatory disorders of seminal vesicle
N49.1 Inflammatory disorders of spermatic cord, tunica vaginalis and vas deferens

Obligate pathogens and viruses are considered the main etiologic factors of combined infection and inflammation process. Most frequently recognized pathogens are *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Mycoplasma genitalium,* but in the last few years there have been many reported the increased disease-causing role of opportunistic microorganisms: *Ureaplasma urealyticum, Ureaplasma parvum, Haemophilus, Candida* etc.

Considering the clinical and social significance of the male genital tract diseases, their slight symptoms or asymptomatic clinical course as well as importance of equivalent medical research of both partners having reproductive system impairment, DNA-Technology Company has developed and implemented the unique technology Androflor® that allows diagnosing combined infection and inflammatory disease of male genital tract (Table 15).

Table 15. Analytes detected by Androflor® REAL-TIME PCR Detection Kit and Androflor® Screen REAL-TIME PCR Detection Kit

Analyte	Androflor® REAL-TIME PCR Detection Kit	Androflor® Screen REAL-TIME PCR Detection Kit
Human DNA	+	+
Total Bacterial Mass (TBM)	+	+
Lactobacillus spp.	+	+
Staphylococcus spp.	+	+
Streptococcus spp.	+	+
Corynebacterium spp.	+	+
Gardnerella vaginalis	+	+
Atopobium cluster	+	–
Megasphaera spp./Veilonella spp./Dialister spp.	+	–
Sneathia spp./Leptotrihia spp. /Fusobacterium spp.	+	–
Ureaplasma urealyticum	+	+
Ureaplasma parvum	+	+
Mycoplasma hominis	+	+
Bacteroides spp./Porphyromonas spp./Prevotella spp.	+	–
Anaerococcus spp.	+	–
Peptostreptococcus spp./Parvimonas spp./Eubacterium spp.	+	–
Pseudomonas aeruginosa/Ralstonia spp./Burkholderia spp.	+	–
Heamophilus spp.	+	–
Enterobacteriaceae/Enterococcus spp.	+	+
Candida spp.	+	+
Mycoplasma genitalium	+	+
Trichomonas vaginalis	+	+
Neisseria gonorrhoeae	+	+
Chlamydia trachomatis	+	+

Androflor® is:

- Detection of infectious agents causing sexually transmitted diseases (STD's)
- Detection of opportunistic microorganisms inhabiting male genital system and having a potency to cause combined infectious-inflammation diseases
- Identification of infectious process's etiology
- Prognosis of therapy intensity
- Dynamic monitoring
- Sample intake control (human genomic DNA quantitative evaluation)

The result interpretation flowchart is shown on figure 10.

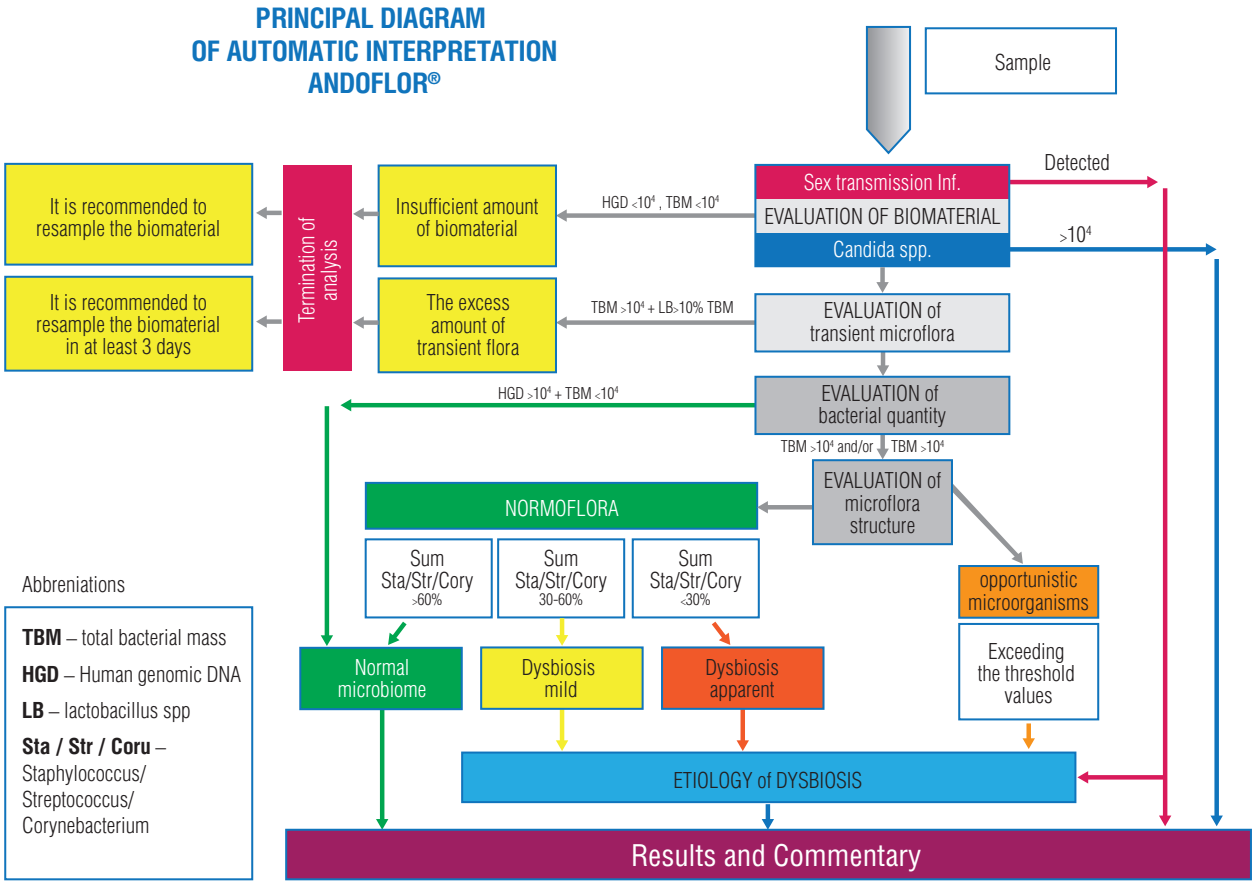


Fig. 10. Androflor® results automated interpretation flowchart

Androflor® intended for (table 16):

- Diagnostics and treatment efficiency monitoring of any combined infection and inflammation diseases of the male genital tract (Androflor® REAL-TIME PCR Detection Kit)
- Diagnostics and treatment monitoring of acute infection and inflammation diseases of the male genital tract (Androflor® Screen REAL-TIME PCR Detection Kit)

Table 16. The Androflor® REAL-TIME PCR Detection Kit and Androflor® Screen REAL-TIME PCR Detection Kit

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Androflor® REAL-TIME PCR Detection Kit	–	–	–	*	RU/IVD
Androflor® Screen REAL-TIME PCR Detection 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-NA-PLUS;*
- *PREP-GS-PLUS.*

Specimen for screening:

- Epithelial cells scrapes from the balanus, urethra; urina; prostatic fluid; ejaculate; biopsy samples from prostatic tissues

ATTENTION! To exclude any biases in microflora composition analysis, caused by transient microflora, the three-day sexual continence or safe sexual contact is obligated before sampling.

- For etiological diagnosis of urethritis, balanoposthitis and therapy monitoring of these diseases the recommended sample materials are urethral scrapes, prepuce of penis scrapes, urine (the first void urine can be used only for pathogens identification).
- For etiological diagnosis of prostatitis, male infertility and therapy monitoring of these diseases the recommended sample materials are prostatic fluid, residual urine after prostate milking, ejaculate and prostate biopsy.

**The prostate milking is strictly prohibited when acute prostatitis is suspected*

Equipment required for analysis:

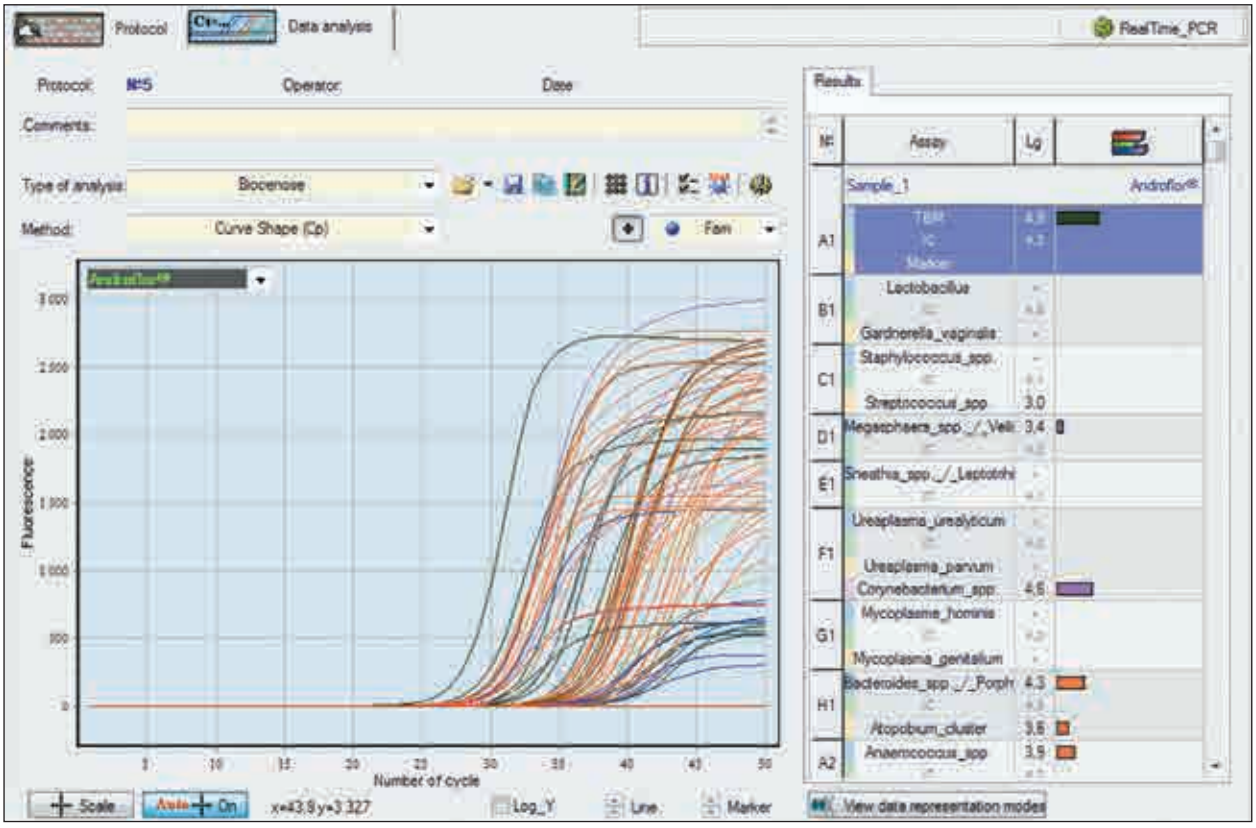
- DT devices produced by DNA-Technology (DTlite, DTprime, DT-96).

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (fig. 11).

A



B

Urogenital microbiome composition test Androflor®

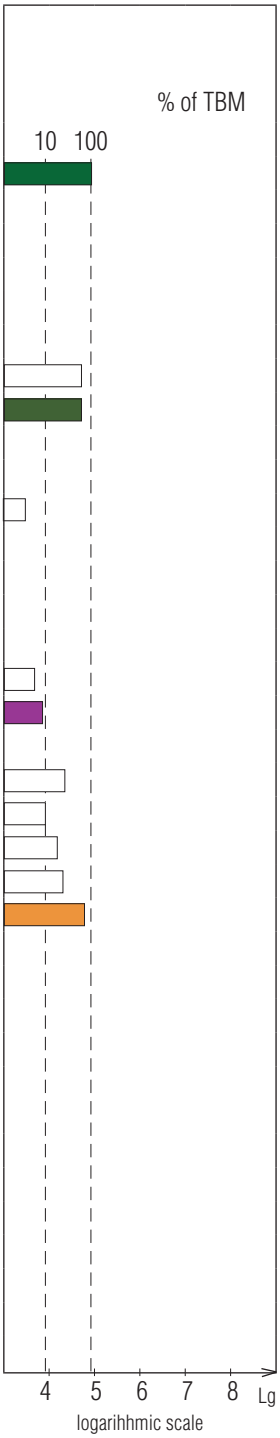
Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

logotype

information about laboratory

Sample ID: Sample_1

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Human DNA	$10^{5,0}$ ☐	
1	Total Bacterial Mass	$10^{4,9}$ ☐	
	Lactobacillus spp.		
2	Lactobacillus spp.	not detected	☐
	Normal microflora		
3	Staphylococcus spp.	not detected	☐
4	Streptococcus spp.	$10^{3,0}$	-1,9 (1,2-1,6 %) ☐
5	Corynebacterium spp.	$10^{4,6}$	-0,3 (46-621 %) ☐
6	Commensals, sum	$10^{4,6}$	-0,3 (47-64 %) ☐
	BV-assoclated opportunistic microorganisms		
7	Gardnerella vaginalis	not detected	☐
8	Megasphaera spp. /Veillonella spp. /Dialister spp.	$10^{3,4}$	-0,9 (10-14 %) ☐
9	Sneathia spp. / Leptotrichia spp. / Fusobacterium spp.	not detected	☐
10	Ureaplasma urealyticum*	not detected ☐	
11	Ureaplasma parvum*	not detected ☐	
12	Mycoplasma hominis*	not detected ☐	
13	Atopobium cluster	$10^{3,6}$	-1,3 (4-5 %) ☐
14	BV-associated opportunistic microorganisns, sum	$10^{3,8}$	-1,1 (7-9 %) ☐
	Opportunistic anaerobes		
15	Bacteroides spp. / Porphyromonas spp. / Prevotella spp.	$10^{4,3}$	-0,6 (21-28 %) ☐
16	Anaerococcus spp.	$10^{3,9}$	-1,0 (9-12 %) ☐
17	Peptostreptococcus spp. / Parvimonas spp.	$10^{4,1}$	-0,8 (14-19 %) ☐
18	Eubacterium spp.	$10^{4,2}$	-0,7 (17-23 %) ☐
19	Opportunistic anaerobes, sum	$10^{4,7}$	-0,1 (61-82 %) ☐
	Opportunistic microorganisms Haemophilus spp.		
20	Haemophilus spp.	not detected	☐
	Opportunistic microorganisms Pseudomonas aeruginosa / Ralstonia spp. / Burkholderia spp.		
21	Pseudomonas aeruginosa / Ralstonia spp. /Burkholderia spp.	not detected	☐
	Opportunistic microorganisms Enterobacteriaceae / Enterococcus spp.		
22	Enterobacteriaceae / Enterococcus spp.	not detected	☐
	Yeast-Like fungl		
23	Candida spp.*	not detected ☐	
	Sexually transmitted infections		
24	Mycoplasma genitalium**	not detected ☐	
25	Trichmonas vaginalis**	not detected ☐	
26	Neisseria gonorrhoeae **	not detected ☐	
27	Chlamydia trachomatis**	not detected ☐	

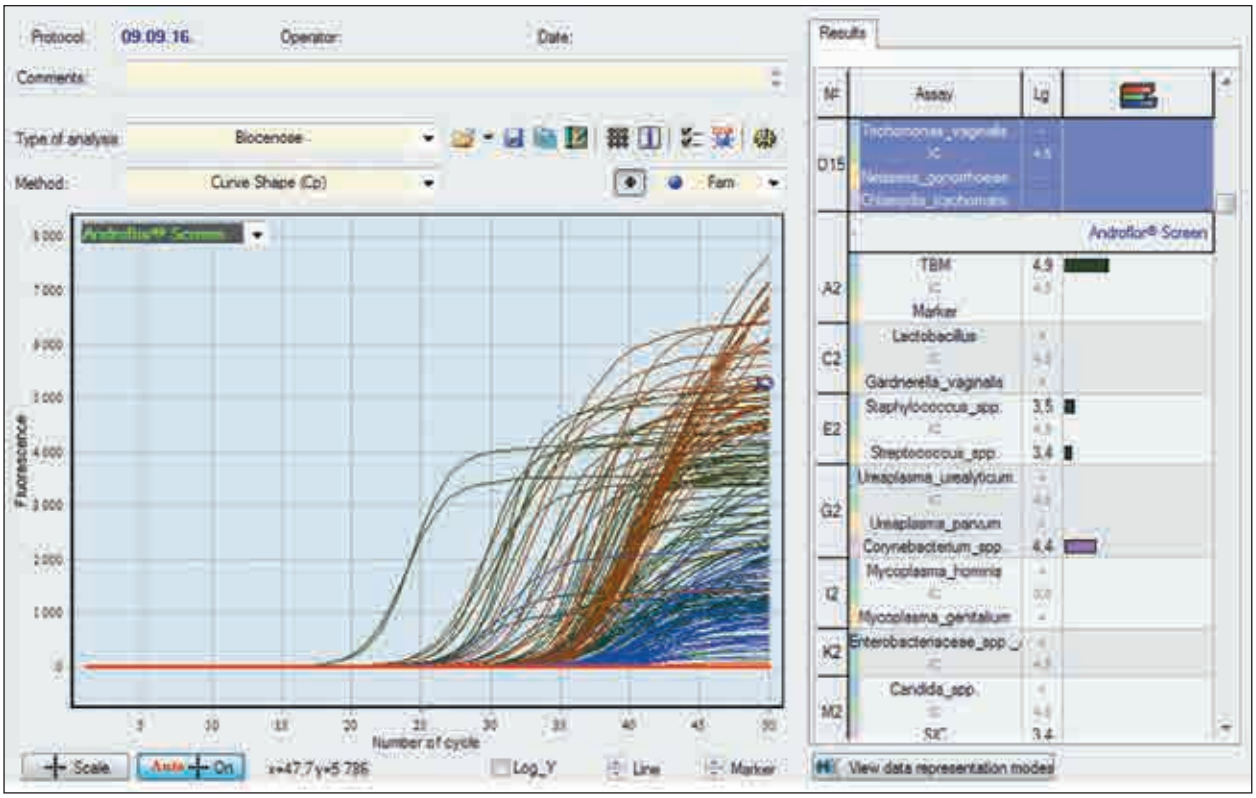


* Quantitative Analysis Lg(X)
** Quantitative Analysis

Conclusiion:

Dysbiosis minor with opportunistic anaerobes predominance

C


Protocol:
09.09.16
Operator:
Date:
Comments:
Type of analysis:
Biocenose
Method:
Curve Shape (Cp)
Fam
Fluorescence
Number of cycle
Scale
Auto On
x=47,7 y=5 786
Log_Y
Line
Marker
Results
Nº
Assay
Lg
D15
Androflor® Screen
A2
TBM
4,9
Marker
C2
Lactobacillus
Gardnerella_vaginalis
E2
Staphylococcus_spp.
3,5
Streptococcus_spp.
3,4
G2
Ureaplasma_urealyticum
Ureaplasma_parvum
Corynebacterium_spp.
4,4
I2
Mycoplasma_hominis
Mycoplasma_genitalium
K2
Enterobacteriaceae_spp._
M2
Candida_spp.
View data representation model

D

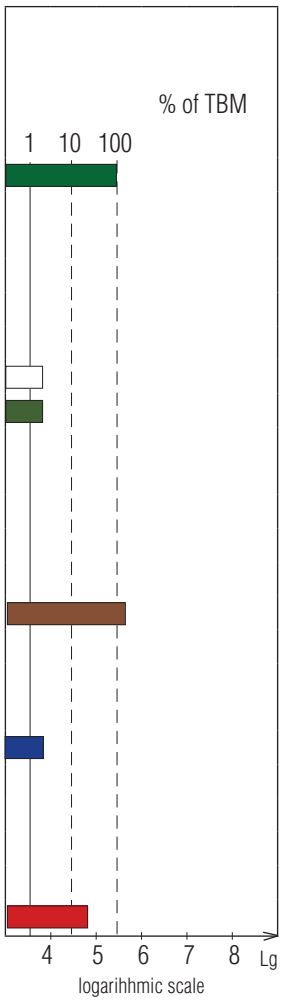
Urogenital microbiome composition test Androflor® Screen

Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

logotype
information about laboratory

Sample ID: -

№	Name of research	Results	
		Quantitative	Relative Lg (O/TBM)
	Human DNA	$10^{4,5}$	
1	Total Bacterial Mass	$10^{5,4}$	
	Lactobacillus spp.		
2	Lactobacillus	not detected	
	Normal microflora		
3	Staphylococcus spp.	not detected	
4	Streptococcus spp.	not detected	
5	Corynebacterium spp.	$10^{3,7}$	-1,6 (1,9-2,6 %)
6	Commensals, sum	$10^{3,7}$	-1,6 (1,9-2,6 %)
	BV-assoclated opportunistic microorganisms		
7	Gardnerella vaginalis	not detected	
8	Ureaplasma urealyticum*	not detected	
9	Ureaplasma parvum*	not detected	
10	Mycoplasma hominis*	not detected	
11	BV-associated opportunistic microorganisns, sum	not detected	0,1-6 (85-100 %)
	Opportunistic microorganisms Enterobacteriaceae / Enterococcus spp.		
12	Enterobacteriaceae / Enterococcus spp.	$10^{5,5}$	
	Yeast-Like fungl		
13	Candida spp.*	$10^{3,6}$	
	Sexually transmitted infections		
14	Mycoplasma genitalium**	not detected	
15	Trichmonas vaginalis**	detected	
16	Neisseria gonorrhoeae **	not detected	
17	Chlamydia trachomatis**	detected	



* Quantitative Analysis Lg(X)
** Quantitative Analysis

Conclusiion:

DYSBIOSIS apparent with Enterobacteriaceae / Enterococcus spp. Detected: Trichomonas vaginalis, Chamydia trachomatis.

Fig. 11. Analysis results for Rt optical measurements (DT devices)
Androflor® REAL-TIME PCR Detection Kit
A – Optical measurement analysis (Fam channel)
B – Form for presentation of results
Androflor® Screen REAL-TIME PCR Detection Kit
C – Optical measurement analysis (Fam channel)
D – Form for presentation of results

10. PARODONTOSCREEN REAL-TIME PCR DETECTION KIT

Microbiocenosis of the oral cavity includes a large number of different types of microorganisms (bacteria, viruses, fungi, protozoa). Microorganisms in the oral cavity comprise both obligate species, persisting in the mouth, and transient ones that appear temporarily, including pathogenic and opportunistic bacteria.

Qualitative and quantitative balance disruptions between normal microflora forms and opportunistic microorganisms are specific to the dysbiosis state, when an increase in the number of potentially pathogenic microorganisms and/or gain in pathogenic power of some microorganisms are observed

Contributing factors of dysbiosis pathogenesis in the oral cavity are: antibiotics, hormonal disorders, changes in the state of local immunity, stress impacts and allergic reactions.

The typical dysbiotic process is chronic **periodontitis**, which is the most common pathology of the oral cavity.

The most important role in parodontium destruction belongs to a group of pathogens in subgingival plaque:

- *Actinobacillus actinomycetemcomitans,*
- *Porphyromonas gingivalis,*
- *Prevotella intermedia,*
- *Tannerella forsythensis,*
- *Treponema denticola.*
- *Candida albicans*

Currently in diagnosing periodontitis the use of traditional methods of clinical and laboratory diagnostics is a common practice. Clinical examination is aimed at identifying complaints and objective clinical symptoms. Laboratory diagnostics is based on detecting etiologically significant pathogen. However, etiologically significant pathogens are mainly anaerobes, which significantly complicate diagnostics because of difficulty of anaerobes cultivation under routine laboratory conditions.

Moreover, qualified clinical examination should be done with focus on defining an etiological factor of the inflammatory process, which could allow adequate etiologically oriented therapy.

An effective solution in diagnosis of dysbiotic disorders in the oral cavity is **ParodontoScreen**, *the technology for quantitative real-time PCR analysis.*

ParodontoScreen allows to detect the main opportunistic microorganisms involved in periodontitis development and performing their quantitative analysis (see Table 17).

Table 17. ParodontoScreen Real-Time PCR Detection Kit

№ of the tube	Cannel			Color of the buffer
	Fam	Hex	Rox	
1	Total bacterial DNA	IC	–	Blue
2	*Actinobacillus actinomycetemcomitans*	IC	–	Colorless
3	*Porphyromonas gingivalis*	IC	–	
4	*Prevotella intermedia*	IC	–	
5	*Tannerella forsythensis (Bacteroides forsythus)*	IC	Marker	
6	*Treponema denticola*	IC	–	
7	*Candida albicans*	IC	–	
8	SIC	IC	–	

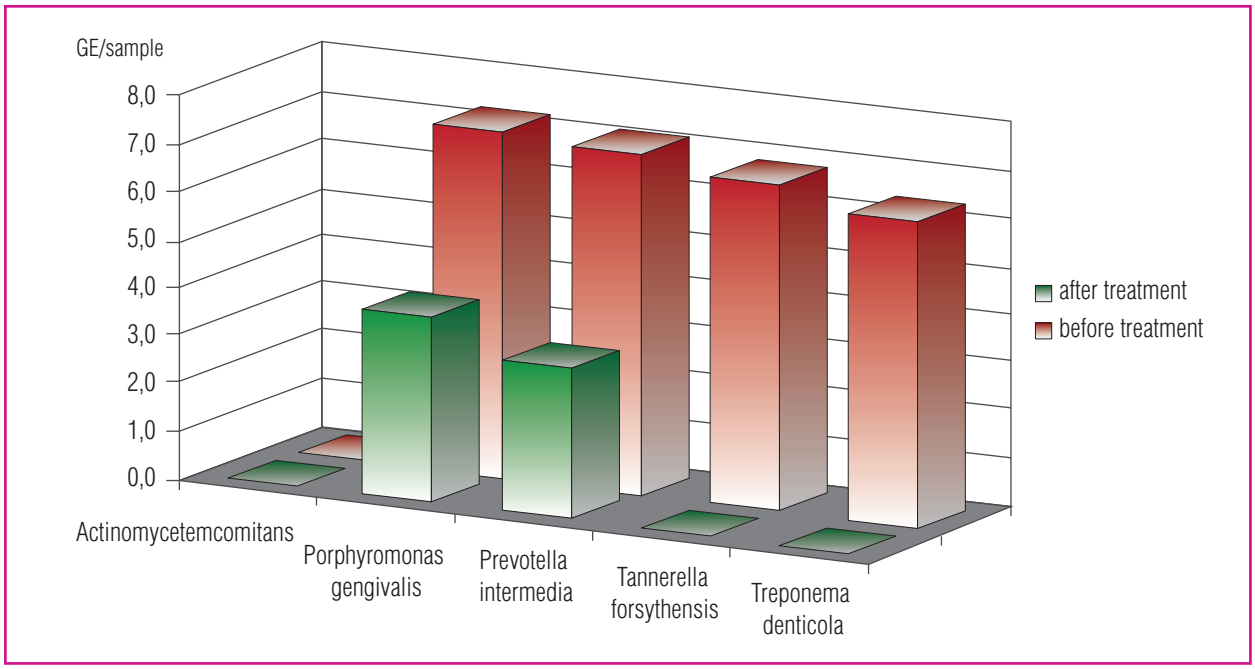
The ParodontoScreen technology allows performing the following tasks within a short time:

- Objectively assess the qualitative and quantitative composition of anaerobic microorganisms simultaneously in different biotopes of the oral cavity
- Monitor oral hygiene
- Optimize disease diagnosis at its initial manifestations
- Conduct differential diagnostics of periodontitis with varying severity of the disease (see Table 18)

Table 18. Interpretation of PCR results based on relative quantity of microorganisms of different clinical evidences and expressed as logarithm (Lg) of genome equivalents per sample

№	Microorganism	Norm	The severity of periodontitis	
			Light/ Moderate	Severe
1	Total bacterial DNA	< 6,5	≥ 6,5	> 7,5
2	*Actinobacillus actinomycetemcomitans*	< 4,0	≥ 4,0	> 5,0
3	*Porphyromonas gingivalis*	< 5,0	≥ 5,0	> 6,0
4	*Prevotella intermedia*	< 4,5	≥ 4,5	> 6,0
5	*Tannerella forsythensis (Bacteroides forsythus)*	< 5,0	≥ 5,0	> 5,5
6	*Treponema denticola*	< 3,5	≥ 3,5	> 5,0
7	*Candida albicans*	< 4,5	≥ 4,5	> 6,0

- Determine the composition of periodontal channels microflora for prescribing etiologically oriented therapy
- Justify selection of antimicrobial therapy
- Monitor the dynamics of changes in anaerobic microorganisms' quantity and species composition during supporting therapy.



Quantity of microorganisms identified in patients with chronic generalized periodontitis and in 1 month after treatment (gingival crevicular fluid).

The technology belongs to direct methods of laboratory analysis – a biomaterial sample is analyzed for presence and quantity of DNA opportunistic microflora. Therefore, it is fundamental to assess the sample intake control (SIC) in terms of controlling the results validity by quantifying the human genomic DNA.

Indications for use:

- Complaints of bleeding gums, other subjective and objective symptoms of inflammation in periodontal tissues
- Diagnosis of severity of the disease and prognosis
- Treatment planning and assessment of effectiveness
- Forecast of disease remission periods
- Assessment of smoking impact on the course of inflammatory process in the periodontium
- Analysis of the state of oral health of healthy individuals

Specimen for screening:

- Saliva
- Gingival crevicular fluid
- Pathological excavation fluid
- Smear containing dental calculus (supragingival dental plaque).

Dentists are advised to take material from the supposed infection localization site. It is a doctor's decision based on a patient's general complaints and a clinical pattern what particular biomaterial should be analyzed for investigating microbiocenosis of the oral cavity or subbiotope.

DNA-Technology offers a kit (see Table 19) for analyzing oral cavity microbiocenosis by PCR method.

Table 19. ParodontoScreen Real-Time PCR Detection Kit

Etiologic agent	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
ParodontoScreen				*	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

Kit format:

- Strip tubes (8 pcs., 0.2 ml each).

Storage temperature: +2 to +8 °C.

Shelf life:

- Rt – 9 months.

DNA extraction kits:

- *PREP-NA-PLUS;*
- *PREP-GS-PLUS.*

Specimen for screening:

- Dental calculus, gingival crevicular fluid.
- Saliva

Equipment required for analysis:

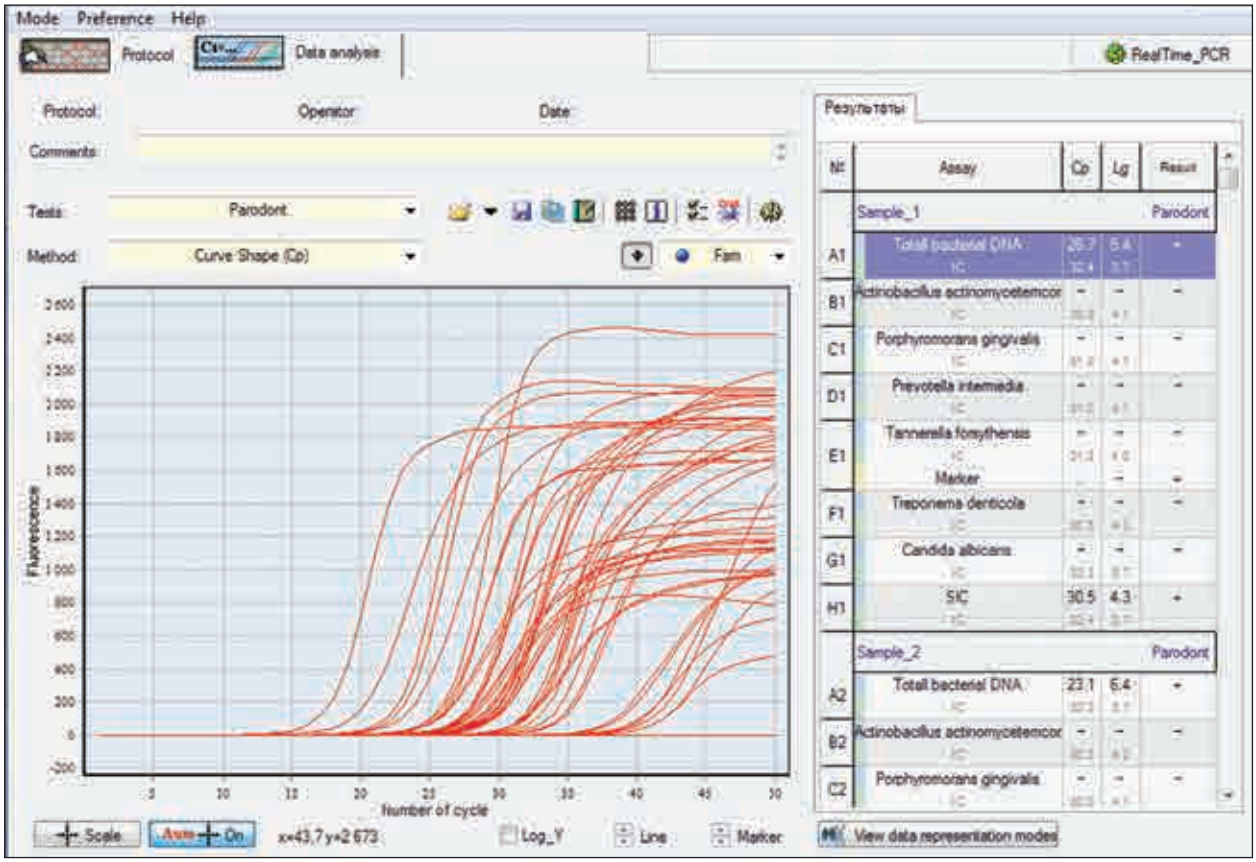
- DT devices produced by DNA-Technology (DTlite, DTprime, DT-96) – with four detection channels and more.

The following additional equipment is needed for analysis using strip tubes:

Strip plastic rack and centrifuge (vortex) rotor.

Software: Reaction results are analyzed and interpreted automatically (for DT devices produced by DNA-Technology) (Fig. 12).

A.



B.

№	Name of research	Results
1	Total bacterial DNA	detected (5.6 Lg)
2	Actinobacillus actonomycetemcomitans	not detected
3	Porphyromorans gingivalis	detected (2.8 Lg)
4	Prevotella intermedi	not detected
5	Tannerella forsythensis	detected (2.1 Lg)
6	Treponema denticola	detected (2.9 Lg)
7	Candida albicans	detected (2.6 Lg)

Fig. 12. ParodontoScreen data readout form in Rt format (DT devices)

A – Optical measurement analysis (Fam channel)

B – Data readout form

IV. PRENATAL DIAGNOSIS

IV. PRENATAL DIAGNOSIS

11. NON-INVASIVE PRENATAL DIAGNOSIS

In current obstetric practice it is often necessary to perform genetic testing of the fetus during early pregnancy. Traditionally, fetal genetic material has been obtained using invasive techniques, such as chorionic villus biopsy or placenta sampling or amnio- and cordocentesis. These procedures, however, carry a risk of miscarriage around 2-3 %. The discovery of cell-free fetal DNA and RNA in maternal blood offered a new source of fetal genetic material for non-invasive prenatal diagnosis. Since material for the research is the mother's blood, these new techniques are safe for the pregnancy compared to the previous methods. The number of fetal DNA in maternal blood increases with increasing of gestational age and depends on the placenta state and characteristics of pregnancy course. Starting from the 8-10 weeks of gestation the methods of non-invasive prenatal genetic testing allow to achieve the level of accuracy 96-100 %.

11.1. Fetal Gender Real-Time PCR Detection Kit

Early determination of fetal gender during first – beginning of the second trimester may help to prevent the birth of children with negative family history. It becomes possible to terminate the pregnancy for medical reasons if the parents have gene variants associated with sex-linked diseases (such as hemophilia or progressive Duchenne/Becker muscular dystrophy in the mother).

Fetal gender must be considered in the appointment of the hormonal therapy to the patients with adrenal hyperandrogenism (congenital adrenal hyperplasia) or other masculinizing endocrine diseases.

The main method of prenatal fetal gender determination is ultrasonography, but it cannot be done reliably in the first trimester of pregnancy because of uncompleted development of the external genitalia and in this regard, ultrasonography can be acknowledged as subjective method.

Indications for the study:

- masculinizing endocrine diseases in pregnant women, including congenital adrenal hyperplasia (CAH); correction of drug therapy.
- genetic variants in pregnant women linked to the sex-associated diseases (hemophilia, X-linked mental retardation, myodystrophy, adrenoleukodystrophy, Alport syndrome, X-linked immunodeficiency, retinitis pigmentosa, X-linked hydrocephalus, Lowe syndrome, X-linked ichthyosis)
- non reliable gender determination by means of ultrasonography.

DNA-Technology Company developed and implemented Fetal Gender REAL-TIME PCR Detection Kit which is intended for detection of cell-free fetal DNA, which derived from multi-copy fragment of Y chromosome, in the blood of pregnant women by Real-Time PCR method (table 20).

The selected target DNA fragment is strictly specific to the Y chromosome in contrast to the SRY gene, which is used as a target in the majority of similar kits of both domestic and foreign manufacturers

Table 20. Fetal Gender REAL-TIME PCR Detection Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Fetal Gender REAL-TIME PCR Detection 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

Method: Multiplex real-time PCR, qualitative analysis

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

Storage temperature: +2 to +8 °C.

Shelf life: Rt – 9 months.

DNA extraction kits:

- *PREP-NA-FET.*

Specimen for screening:

Peripheral blood.

Equipment required for analysis:

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

The following additional equipment is needed for analysis using strip tubes:

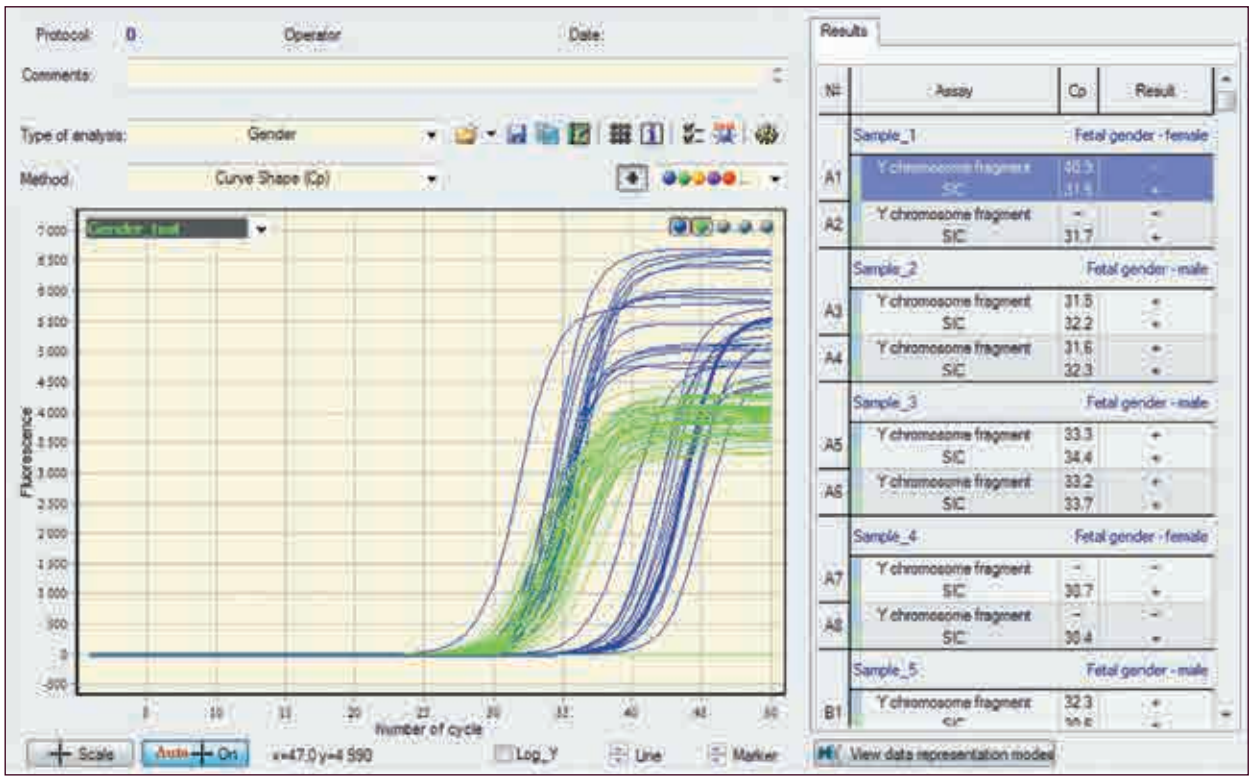
- strip plastic rack and centrifuge (vortex) rotor;
- tube rack and vortex adaptor suitable for stripped PCR tubes;
- cooling tube rack (is necessary during the DNA extraction step!)

Features of the Kit:

- Multiplex analysis – simultaneous detection of multiple targets in the one tube (Y chromosome fragment and human genomic DNA)
- SIC is intended for extraction quality assessment as well as for evaluation of sufficiency of sample for obtaining reliable result.
- Due to the small amount of fetal DNA in the blood of pregnant women analysis of each DNA sample **must be done in duplicate**. Otherwise the results will not be obtained.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (fig. 13).

A



B

	Sample_4	Fetal gender – female	
A7	Y-chromosome fragment	–	–
	SIC	30.7	+
A8	Y-chromosome fragment	–	–
	SIC	30.4	+

Prenatal diagnostics.
Fetal gender.

Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

logotype

information about laboratory

Sample ID: Sample_4

Name of research	Result	Interpretation of result
Detection of Y-chromosome fragment	Not detected	Fetal gender – female.

Attention: Precision of test is 96-100 % at over 8 weeks gestationalage.

Study was carried out by

Data
Signature

C

	Sample_5		Fetal gender – male	
B1		Y-chromosome fragment	32.3	+
		SIC	30.6	+
B2		Y-chromosome fragment	32.1	+
		SIC	30.6	+

Prenatal diagnostics.
Fetal gender.

Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

logotype

information about laboratory

Sample ID: Sample_5

Name of research	Result	Interpretation of result
Detection of Y-chromosome fragment	Detected	Fetal gender – male.

Attention: Precision of test is 96-100 % at over 8 weeks gestationalage.

Study was carried out by

Data
Signature

Fig. 13. Analysis results for Rt optical measurements (DT devices)
A – Optical measurement analysis (Fam channel)
B – Form for presentation of results (Fetal gender – Female)
C – Form for presentation of results (Fetal gender – Male)

When doubtful or non reliable results are obtained, analysis must be repeated according to the proposed algorithm (Table 21).

Table 21. Definition of doubtful and non reliable results

Results on the FAM channel (FAM Cp)	Results on the HEX channel (HEX Cp)	Interpretation
Is not considered	Cp>35 or is not specified	Non reliable result**
Results interpretation does not match in duplicates	Cp≤35	Non reliable result*
35<Cp≤37	Cp≤35	Doubtful result*

* PCR amplification must be repeated

** PCR amplification must be repeated or DNA extraction and PCR amplification must be repeated or blood sampling, DNA extraction and PCR amplification must be repeated (performed sequantially).

11.2. Fetal RHD Genotyping Real-Time PCR Kit

There are five antigens in Rh system. The most immunogenic is D-antigen, the presence of which on erythrocyte surface defines positive Rh factor (Rh+). Portion of antigen D carriers in population reaches 86 % and rhesus negative people (Rh-) who lack antigen D is around 14 %. The cases when Rh- woman is pregnant with Rh+ fetus often complicated with development of hemolytic disease of newborn or fetal erythroblastosis, associated with transplacental passage of fetal erythrocytes to maternal blood stream. The 98 % of hemolytic disease of newborn associated with D-antigen. Upon getting into Rh- mother's blood, D-antigen causes formation of specific antibodies, which cross the placenta and destroy fetal erythrocytes, provoking the development of hemolytic disease of newborn. Early manifestation of Rh disease can cause premature birth or spontaneous abortion. The maternal sensibilization to D-antigen and risk of Rh disease development increase with every subsequent pregnancy with Rh+ fetus, independently of whether abortion was applied or successful delivery was achieved.

The standard methods of Rh incompatibility evaluation include costly and time-consuming assays:

- Evaluation of maternal specific antibodies to fetal D-antigen
- Invasive approaches based on obtaining of fetal sample, like chorionic villus biopsy or placenta sampling or amnio- and cordocentesis.
- Doppler velocimetry based evaluation of blood flow velocity in medial cerebral artery and aorta of the fetus.

All Rh- pregnant women passing dynamic control of antibodies level to fetal D-antigen. The absence of antibodies cannot guarantee that fetus is also Rh- because production of maternal antibodies can be inhibited due to integrity of placenta or suppressed immune response. Risk of spontaneous Rh disease development remains under given circumstances. Moreover, mother will be sensitized in the course of delivery.

DNA-Technology's Fetal RHD Genotyping REAL-TIME PCR Kit is intended for detection of fetal RHD gene from maternal blood by real-time PCR method and allows to evaluate Rh state of fetus at early gestational age in Rh- female. This information is helpful for early Rh disease risk detection and implementation of preventive measures. The kit can be used in framework of prenatal screening programs aimed at prevention of pregnancy complications in Rh- women.

Rh factor testing

Testing of Rh factor by real-time PCR method is based on detection of RHD gene encoding D-antigen. Classic serological method is based on direct detection of D-antigen on a surface of erythrocytes. Most commonly negative Rh factor is defined by complete deletion of RHD gene. In this case, both methods detect negative Rh factor.

However in 1 % of serologically Rh- people RHD gene is present. It is caused due to the following reasons:

- RHD gene is present and genetically Rh factor is considered positive, but, due to the mutations, gene is not expressed and D-antigen is not produced, so serologically Rh factor is defined as negative.
- RHD gene is present in full or partially and genetically Rh factor is considered positive, but, due to the mutations, gene is expressed alternatively and D-antigen produced in a modified form, so serologically Rh factor is defined as weak or unstably positive.

Such patients are genetically Rh+ and PCR based detection of fetal Rh factor is impossible. However, the pregnancy follow-up should be conducted in accordance with care regimen specific for Rh- patients with risk of Rh disease development.

Indications for the study:

- Follow-up of Rh- female pregnancy, allowing estimation of Rh disease development risk.
- Absence of antibodies to fetal D-antigen in blood of Rh- mother before prophylactic administration of immunoglobulin
- Surgical abortion in Rh- female, allowing estimation of Rh disease development in subsequent pregnancies.

Intended use of the kit

Fetal RHD Genotyping REAL-TIME PCR Kit is intended for detection of fetal RHD gene based on specific detection of two exons of RHD gene (7th and 10th) in the blood of Rh- mother. The two exon detection covers big heterogenic group of gene variants and thus raises reliability of results.

DNA-Technology's Fetal RHD Genotyping REAL-TIME PCR Kit intended for detection of fetal RHD gene from maternal blood by real-time PCR method, is an innovative product allowing identification of genetically Rh+ patients (table 22).

Table 22. Fetal Gender REAL-TIME PCR Detection Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
Fetal RHD Genotyping Real-Time PCR 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

Method: Multiplex real-time PCR, qualitative analysis

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-NA-FET.*

Specimen for screening: Peripheral blood.

Equipment required for analysis:

- For Rt kit – DT devices produced by DNA-Technology (DTlite, DTprime, DT-96).

The following additional equipment is needed for analysis using strip tubes:

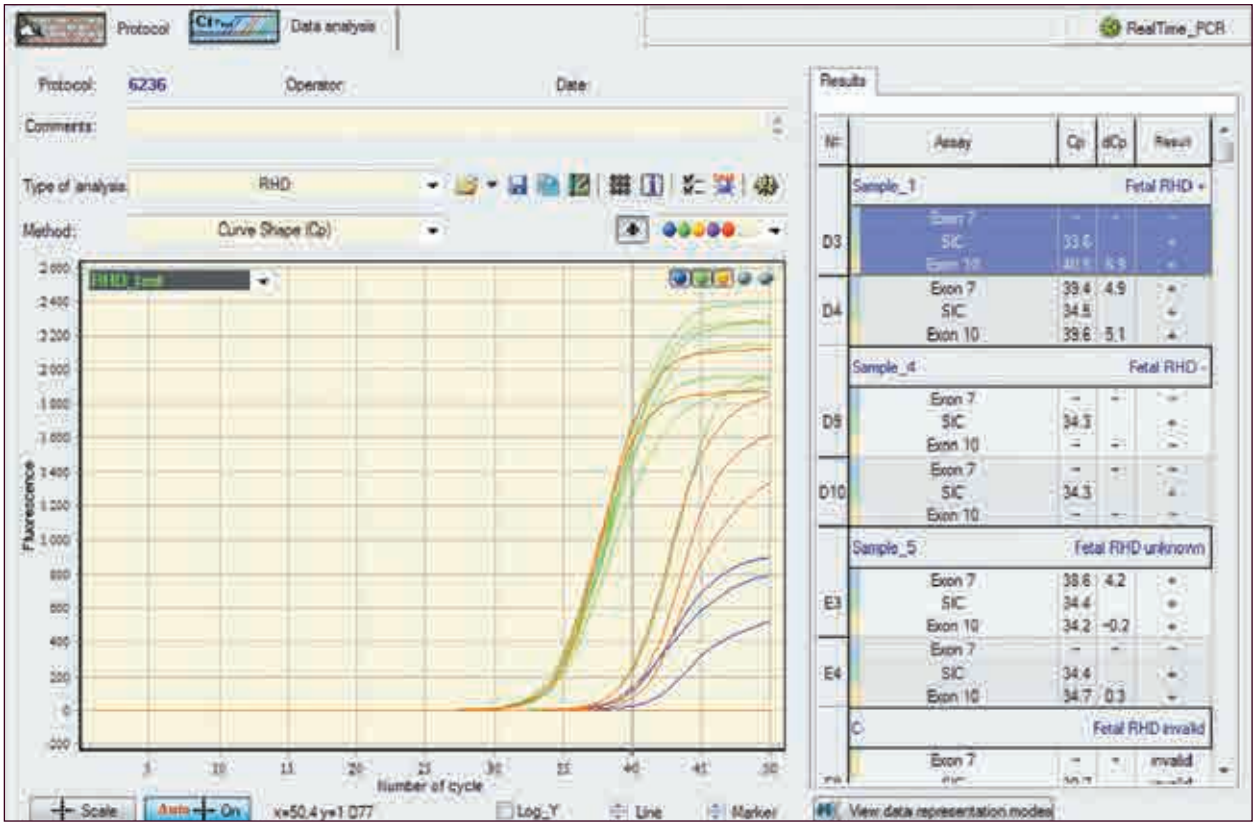
- strip plastic rack and centrifuge (vortex) rotor;
- tube rack and vortex adaptor suitable for stripped PCR tubes;
- cooling tube rack (is necessary during the DNA extraction step!)

Features of the Kit:

- Multiplex analysis – simultaneous detection of multiple targets in the one tube – 7th and 10th exons of RHD gene and human genomic DNA (SIC)
- SIC is intended for extraction quality assessment as well as for evaluation of sufficiency of sample for obtaining reliable result.
- Due to the small amount of fetal DNA in the blood of pregnant women analysis of each DNA sample **must be done in duplicate**. Otherwise can be obtained incorrect results.

Software: Reaction results are analyzed and interpreted automatically (for devices produced by DNA-Technology) (fig. 14).

A



B

		Sample_4	Fetal RHD –		
D9		Exon 7	–	–	–
		SIC	34,3		+
		Exon 10	–	–	–
D10		Exon 7	–	–	–
		SIC	34,3		+
		Exon 10	–	–	–

Prenatal diagnostics.
Fetal RHD Genotyping.

logotype

Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

information about laboratory

Sample ID: Sample_4

Name of research	Result	Interpretation of result
Fetal RHD Genotyping	Not detected	Rh factor of fetus is: genotypically negative

Attention: Precision of test is 96-100 % at over 8 weeks pregnancy in serologically rhesus negative patient.

Conclusion:

Development of rhesus conflict is unikely (less than 0.1%)

The precision of test depends on fetal DNA quantity, detected in female plasma. This value is determined by condition of placenta and raises with the progression of pregnancy.

The detection of rhesus factor by real-time pcr is based on specific detection of RHD gene, coding D-antigen in female blood.

Traditional serological method is based on direct detection of D-antigen expressed on red blood cells.

Normaly negative rhesus factor is defined by absence of RHD gene, and therefore absence of D-antigen. In this case both methods show negative rhesus factor, so results coincide.

In very rare cases (less than 0.1%) fetal RHD gene is genotypically detected as negative, bu after birth serologically detected as weakly-positive. In this case weak rhesus conflict is possible.

C

	Sample_1		Fetal RHD +		
D3		Exon 7	–	–	–
		SIC	33,6		+
		Exon 10	4,5	6,9	+
D4		Exon 7	39,4	4,9	+
		SIC	34,5		+
		Exon 10	39,6	5,1	+

Prenatal diagnostics.
Fetal RHD Genotyping.

logotype

Date
Nomber tube
Patient name
Sex
Age
Organization
Clinician name
Comments

information about laboratory

Sample ID: Sample_1

Name of research	Result	Interpretation of result
Fetal RHD Genotyping	Detected	Rh factor of fetus is: genotypically positive

Attention! The precision of test is 96-100 % at later than 8th weeks pregnancy in serologically rhesus negative patient.

Conclusion:
Development of rhesus conflict is possible.

The precision of test depends on fetal DNA quantity, detected in female plasma. This value is determined by condition of placenta and raises with the progression of pregnancy.

The detection of rhesus factor by real-time pcr is based on specific detection of RHD gene, coding D-antigen in female blood.

Traditional serological method is based on direct detection of D-antigen expressed on red blood cells.

In 99% result of serological and genetic tests are correlate. However in 1% of genotypicaly rhesus positive people are serologically rhesus negative. This could be associated with mutations in RHD gene which cause absence or functioal deficiency of D-antigen. So after dirth we recommend serological reconfirming of rhesus factor.

Fig. 14. Analysis results for Rt optical measurements (DT devices)
A – Optical measurement analysis (Fam channel)
B – Form for presentation of results (Fetal RHD-)
C – Form for presentation of results (Fetal RHD+)

When doubtful or non reliable results are obtained, analysis must be repeated according to the proposed algorithm (see Table 23).

Table 23. Definition of doubtful and non reliable results

Parameters of test	Variants		
	1	2	3
FAM channel (Fam Cp)	Cp 41	Not considered	Not considered
HEX channel (Hex Cp)	Cp 35	Cp>35 or is not specified	Cp 35
ROX channel (Rox Cp)	Cp 41	Not considered	Not considered
ΔCp (Fam Cp – Hex Cp)	1,0-1,9	Not considered	Not considered
ΔCp (Rox Cp – Hex Cp)	1,0-1,9	Not considered	Not considered
Result of amplification	+	N/A	Result does not match in duplicates
Result of test (fetal RGD gene)	?	N/A	N/A
Interpretation	Doubtful result*	Non reliable result***	Non reliable result***

*** Note:**

* PCR amplification, DNA extraction and PCR amplification or sampling must be repeated (performed sequentially)

** DNA extraction and PCR amplification or sampling must be repeated (performed sequentially).

*** PCR amplification must be repeated

IV. HUMAN GENETICS

IV. HUMAN GENETICS

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).

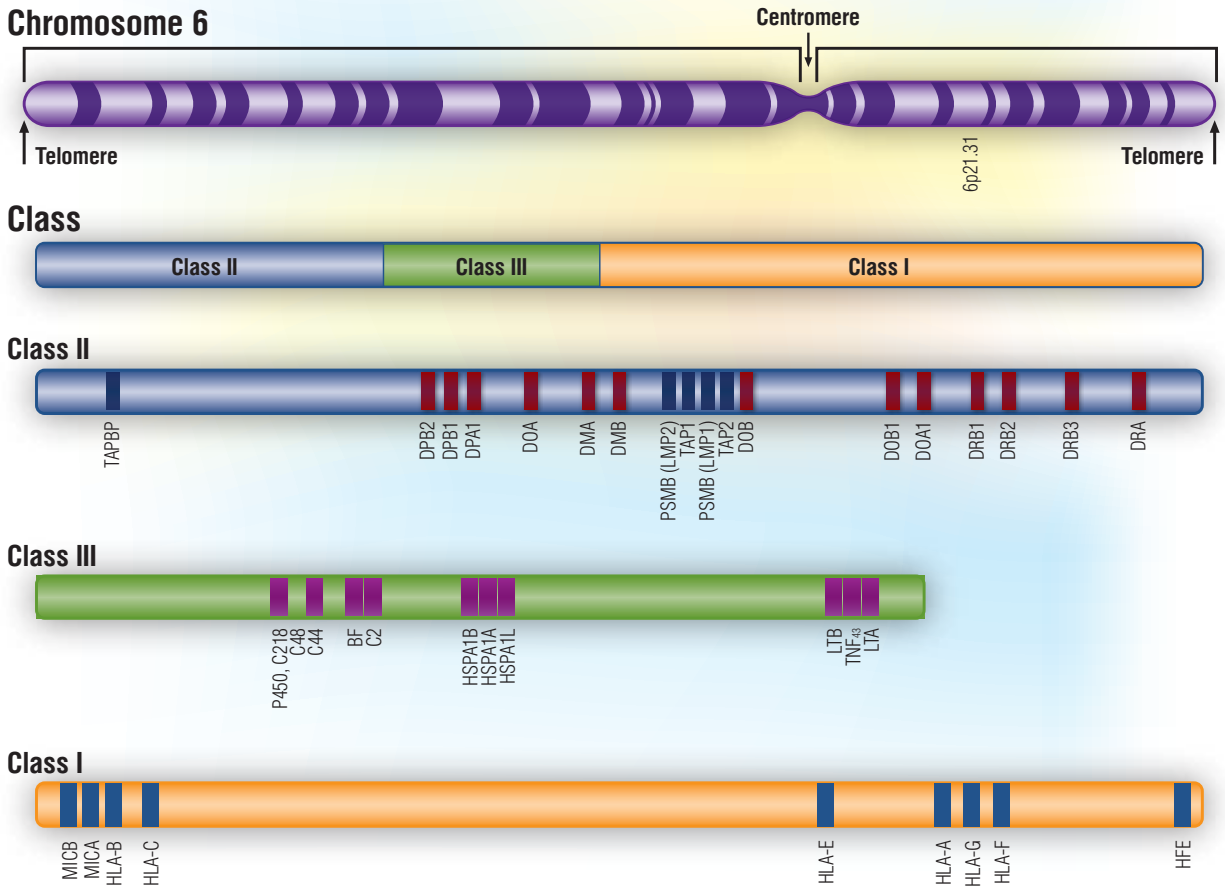


Fig. 15. Major histocompatibility complex (HLA)

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.

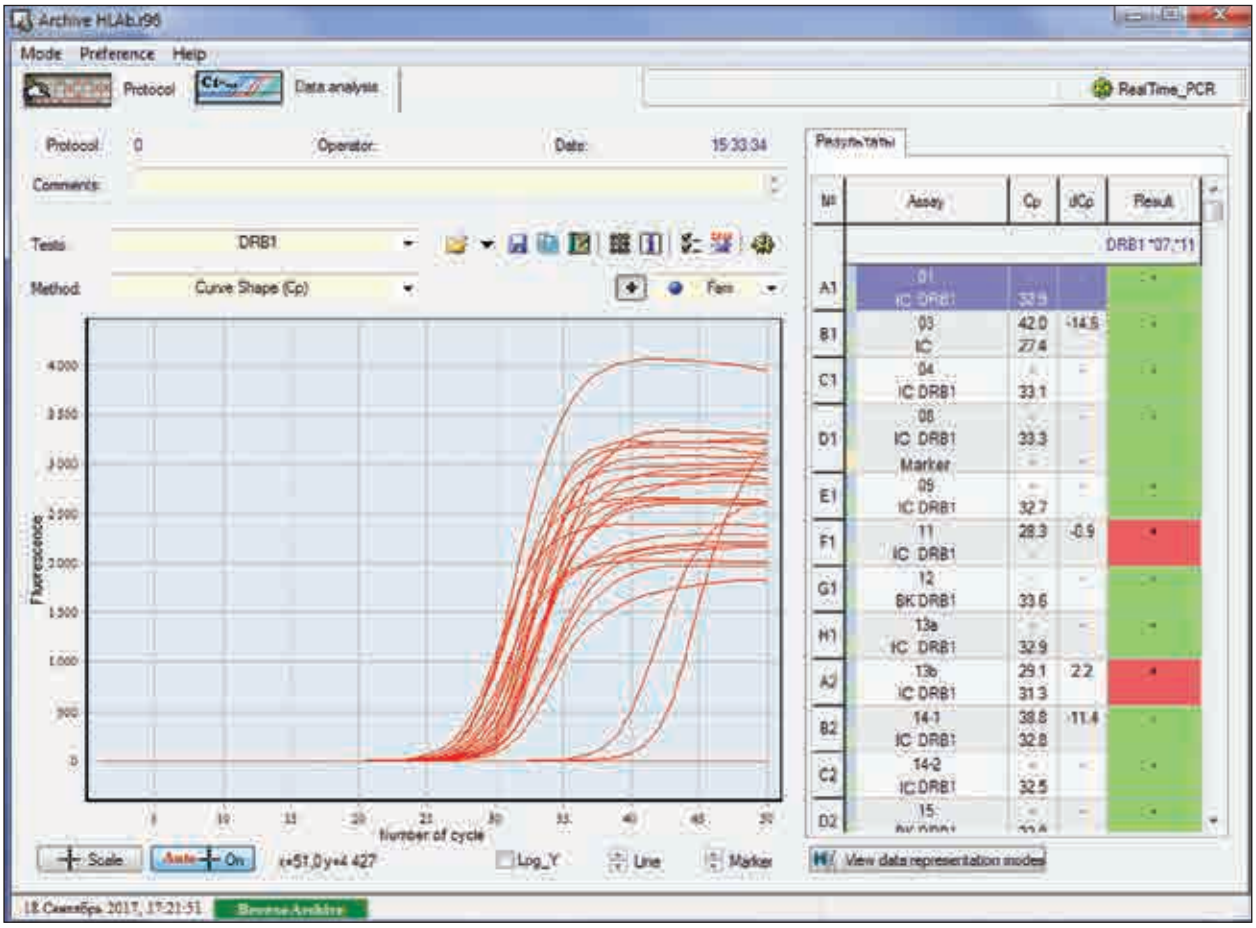
Equipment required for analysis:

- 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).

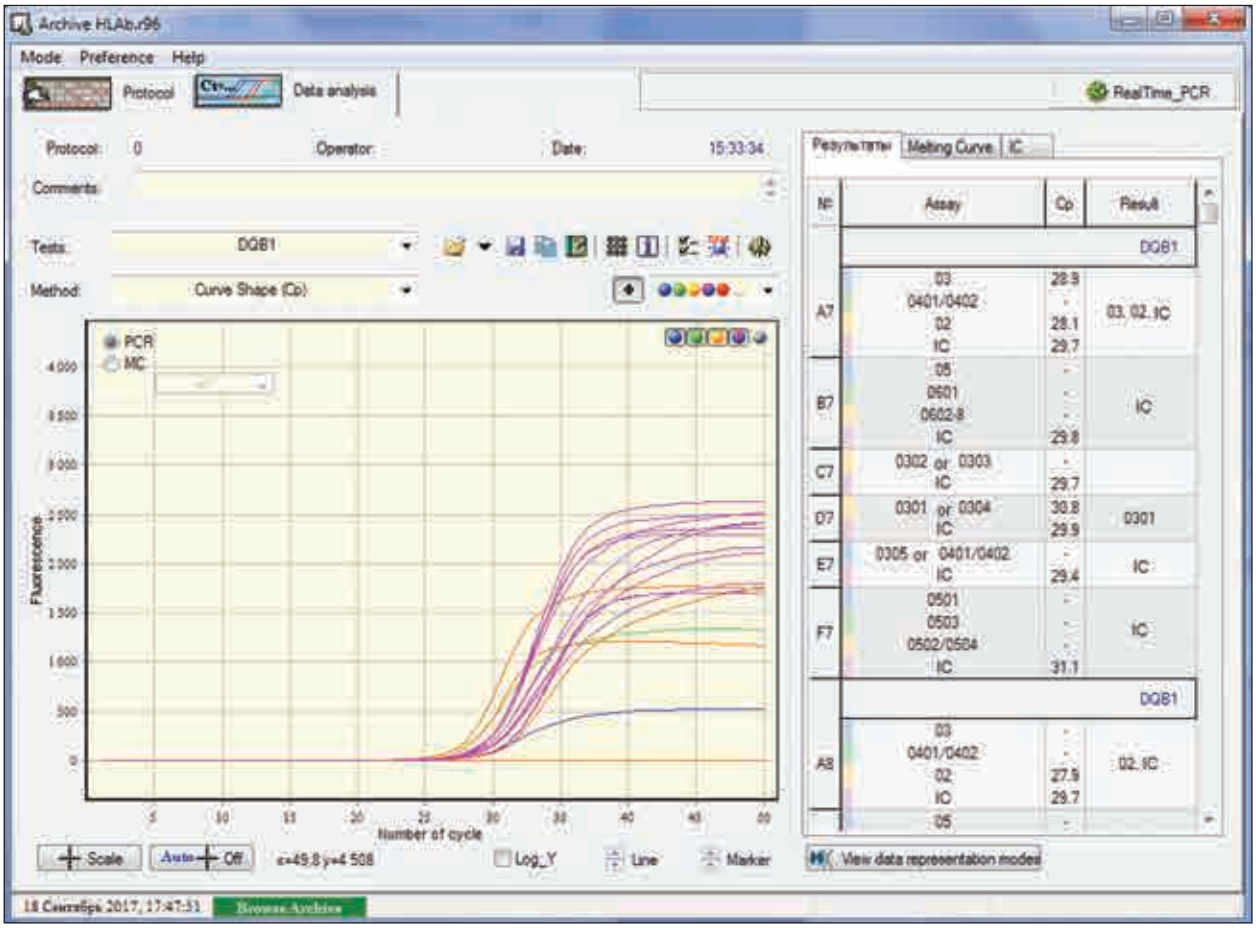
A



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.

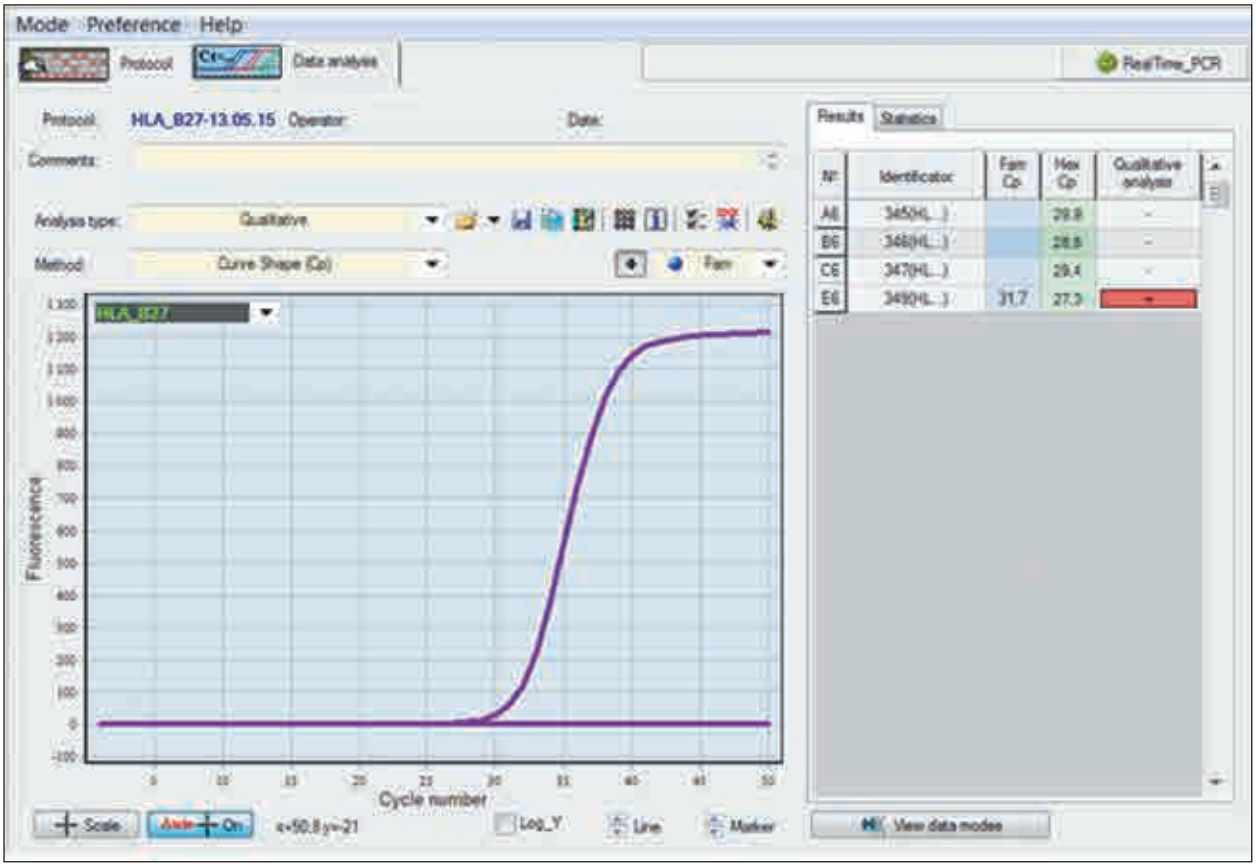
Equipment required for analysis:

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).

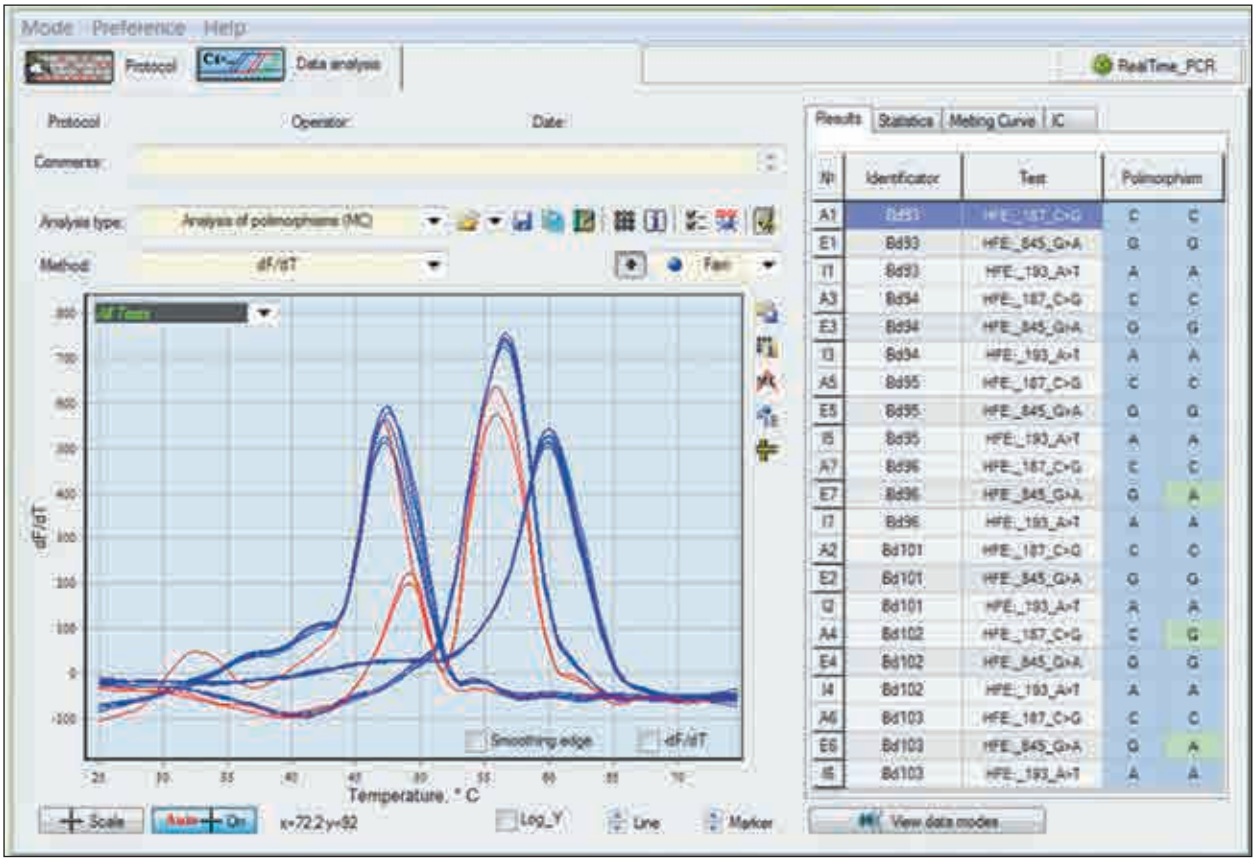
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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

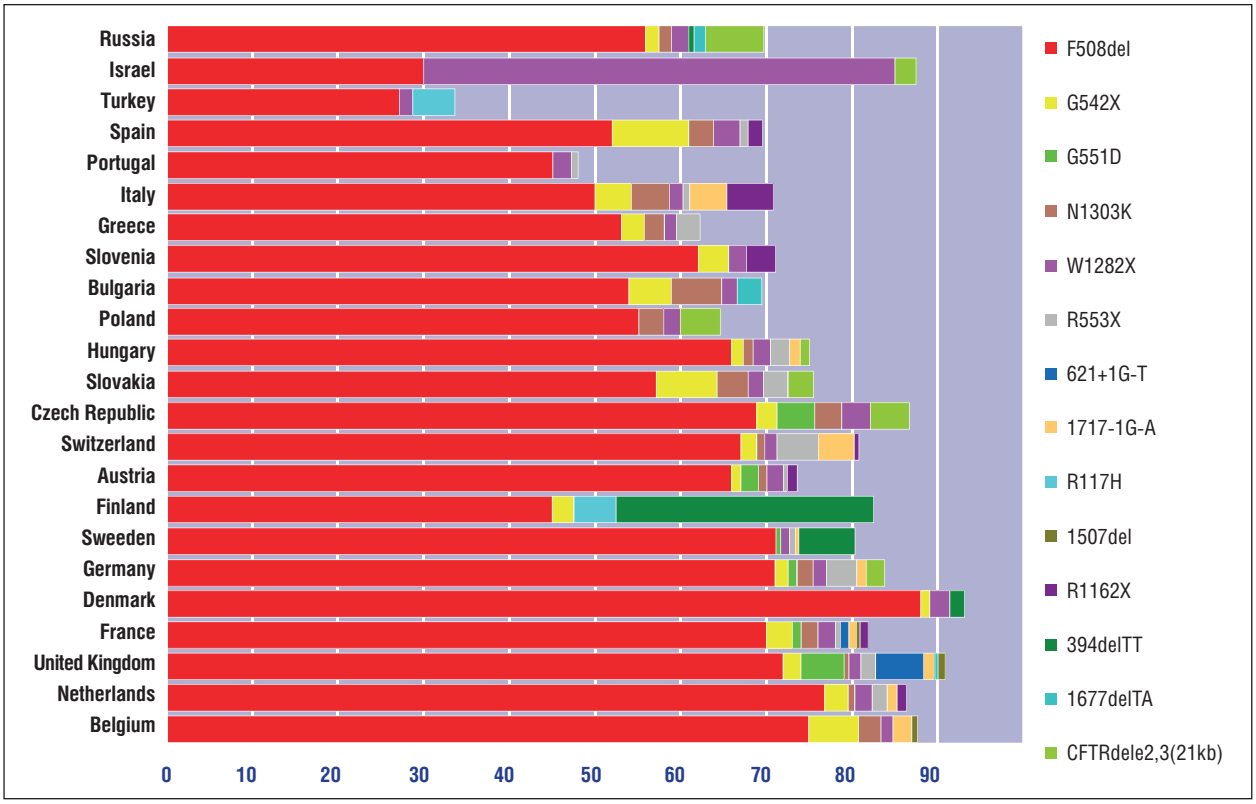


Fig. 19. Relative frequency of CFTR mutations in some populations of Eurasia (Petrova N.V., 2010)

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).

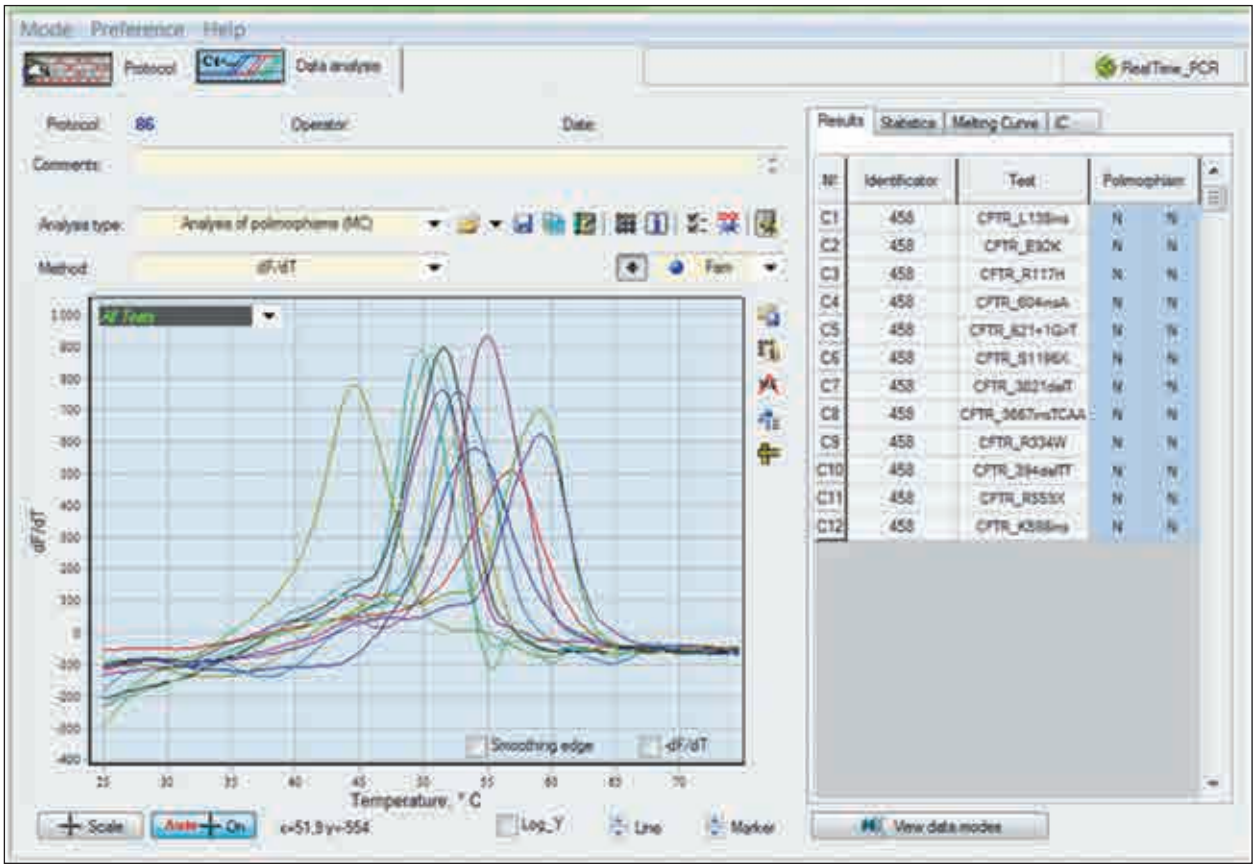
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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.

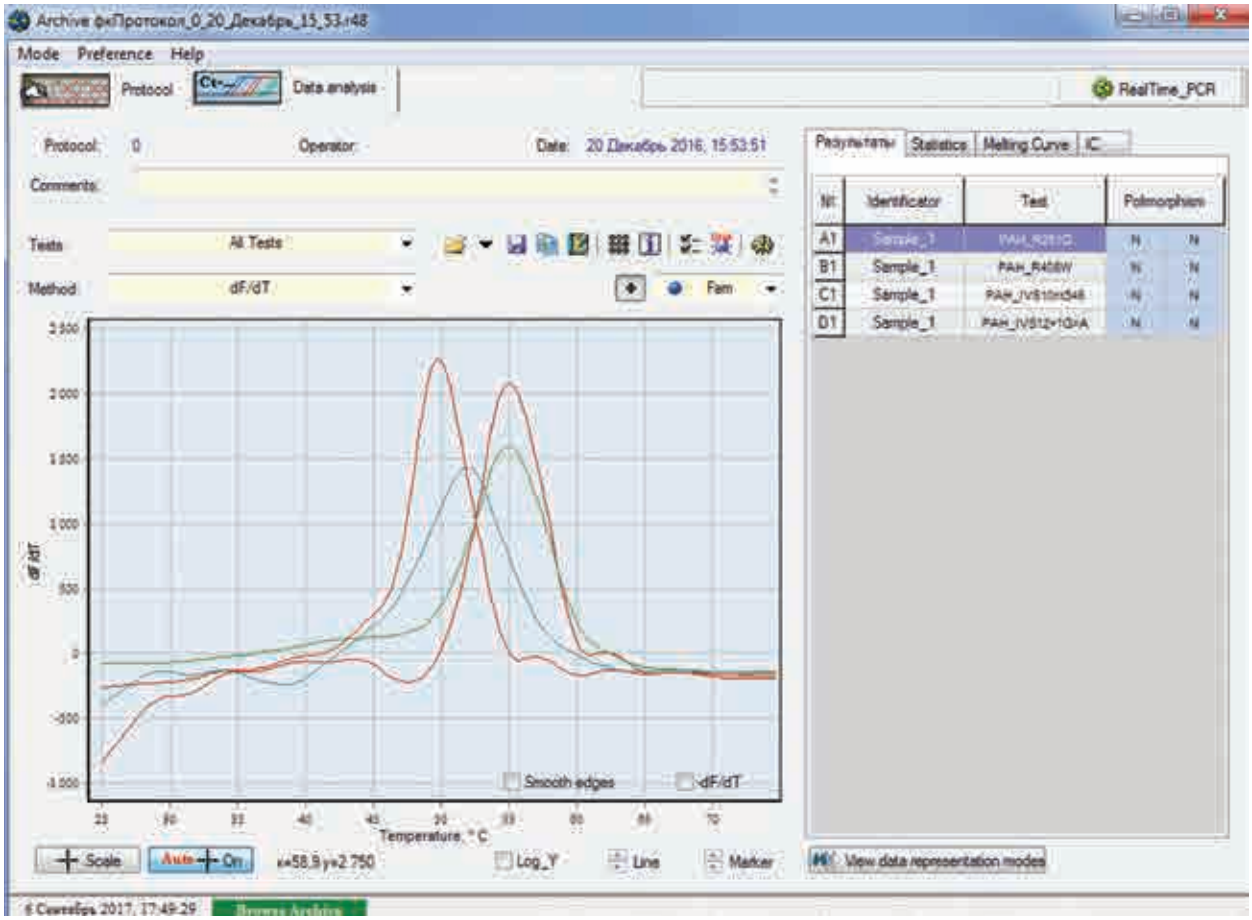
The following equipment and supplies are required for the analysis:
- 0.2 ml microtubes (or strips) for PCR analysis, adapted for use with thermal cycler in real-time;
- Strip plastic rack and centrifuge (vortex) rotor.

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.

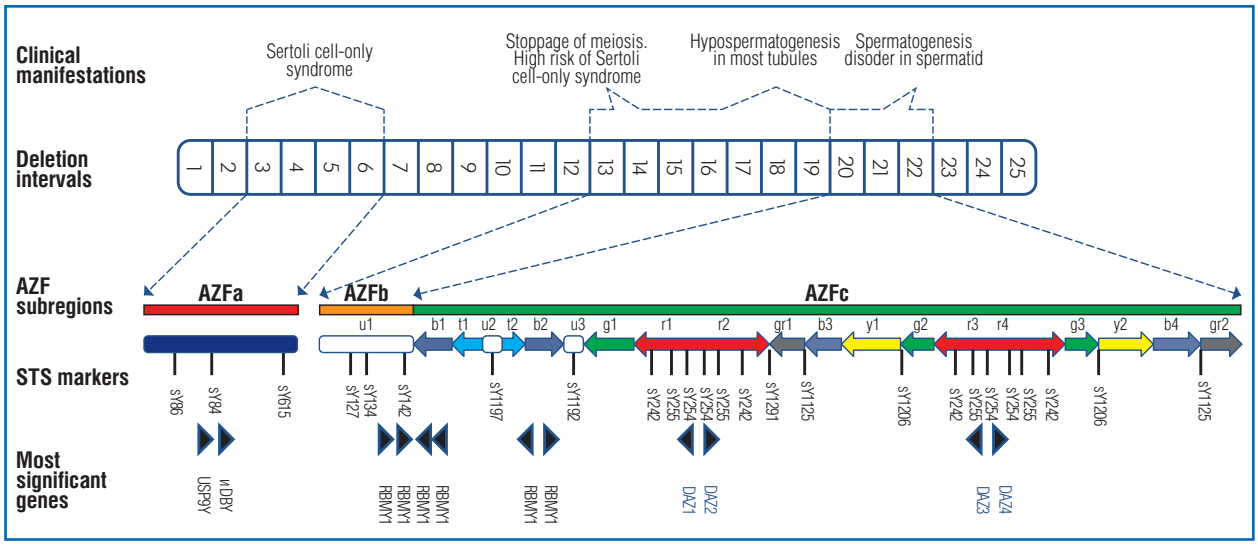


Fig. 22. Schematic representation of the AZF region

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 3.5×10^6 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].

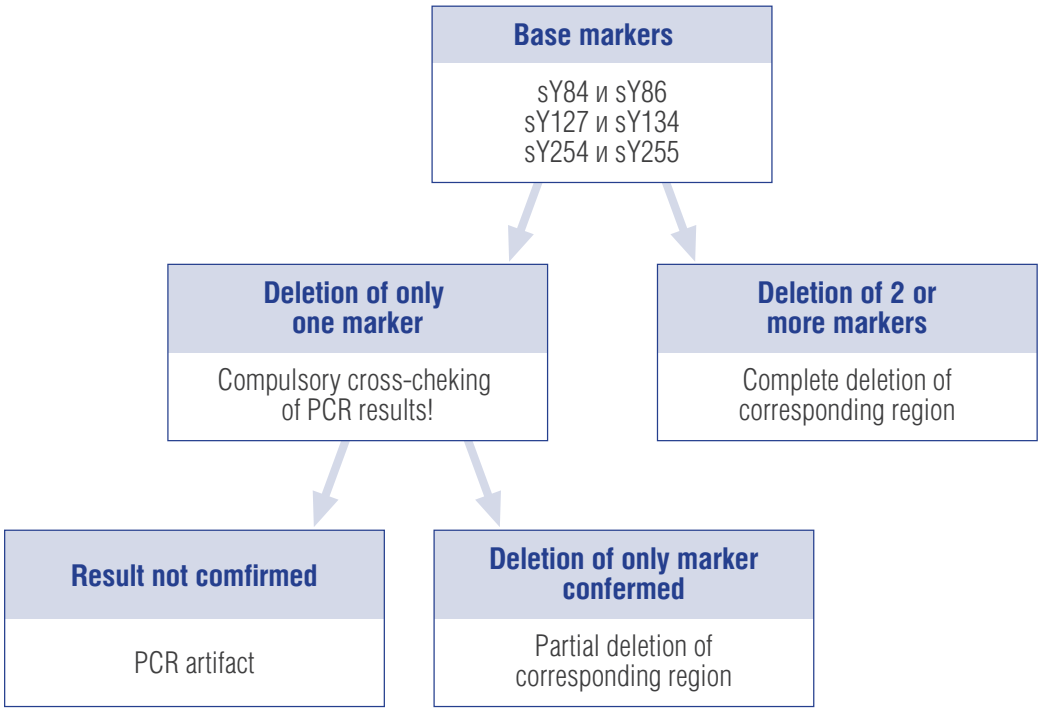


Fig. 23. Block diagram for lab self-control, with indication of analytical steps (Krausz C., 2014)

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:

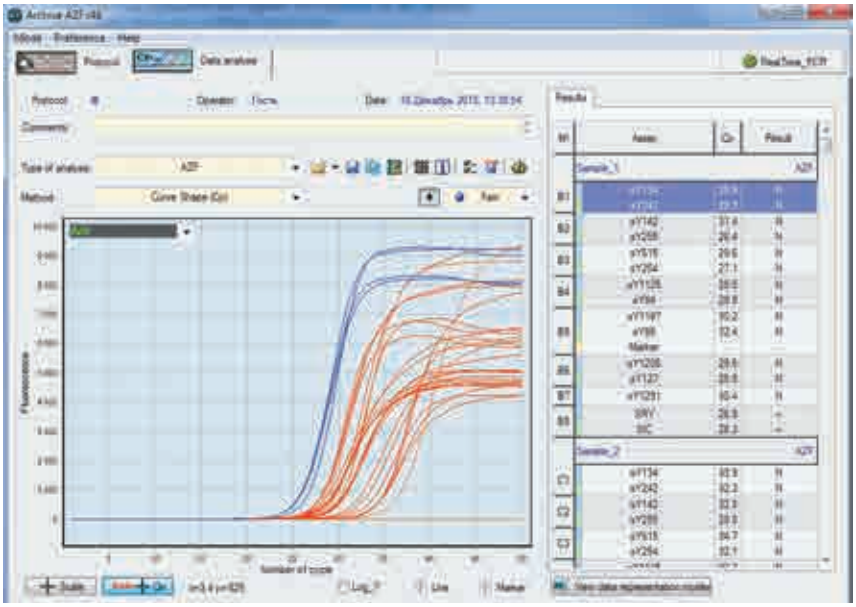
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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).

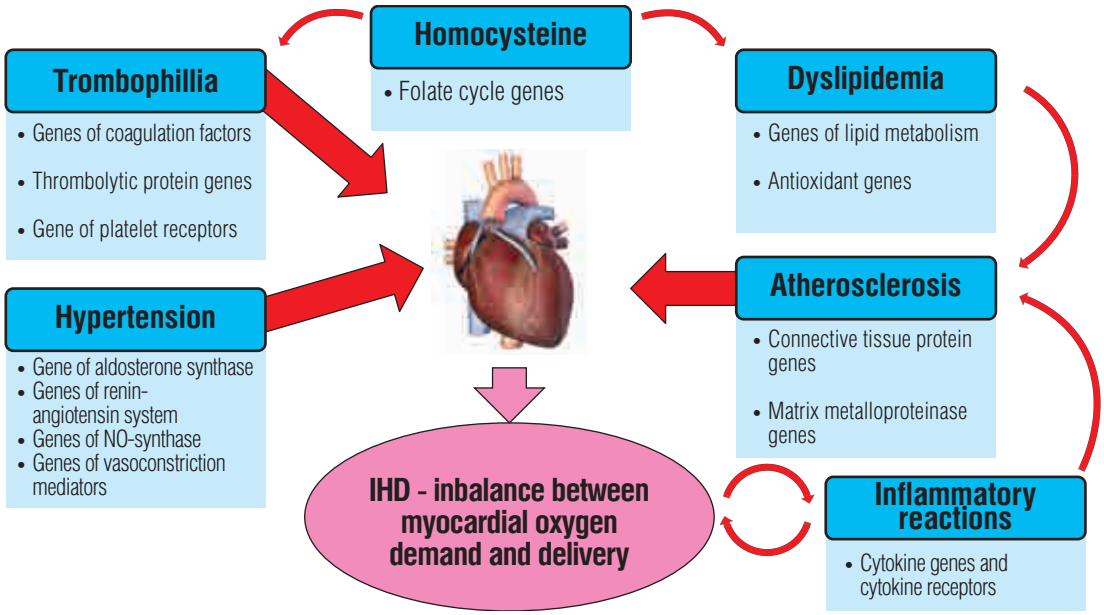


Fig. 25. Gene network. Cardiogenetics. Ischemic heart disease

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.

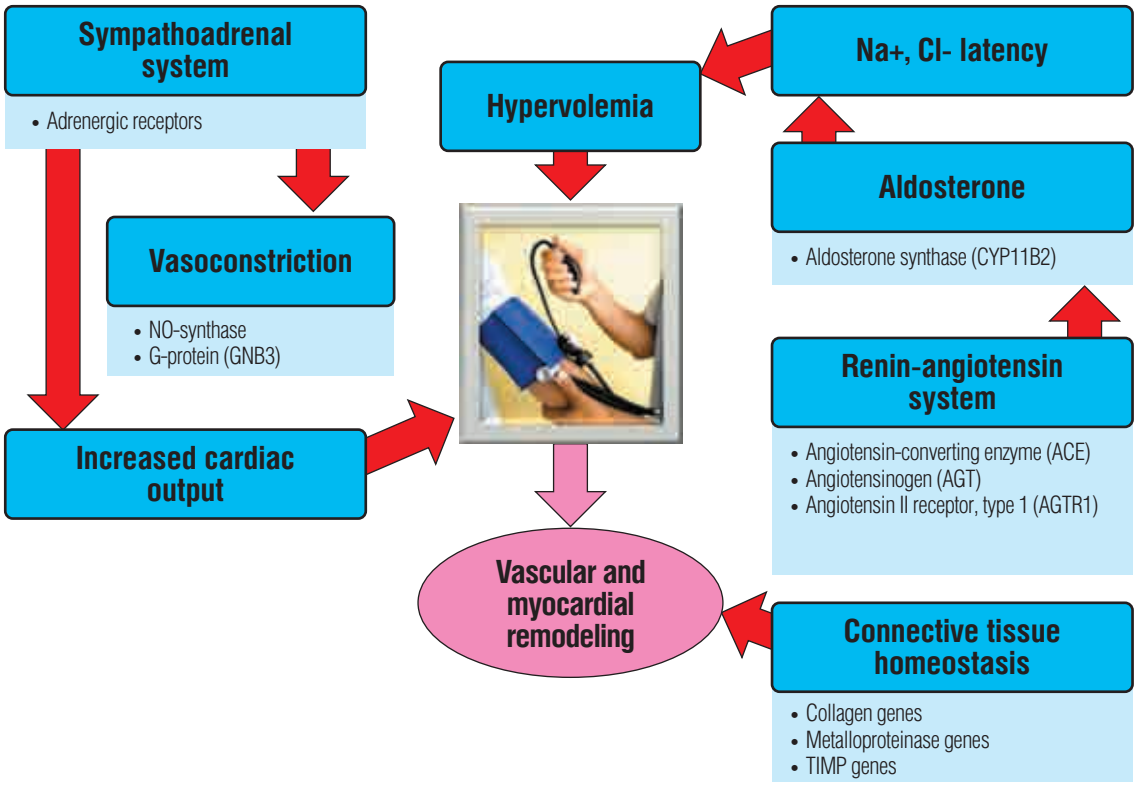


Fig. 26. Gene network. Cardiogenetics. Hypertention

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).

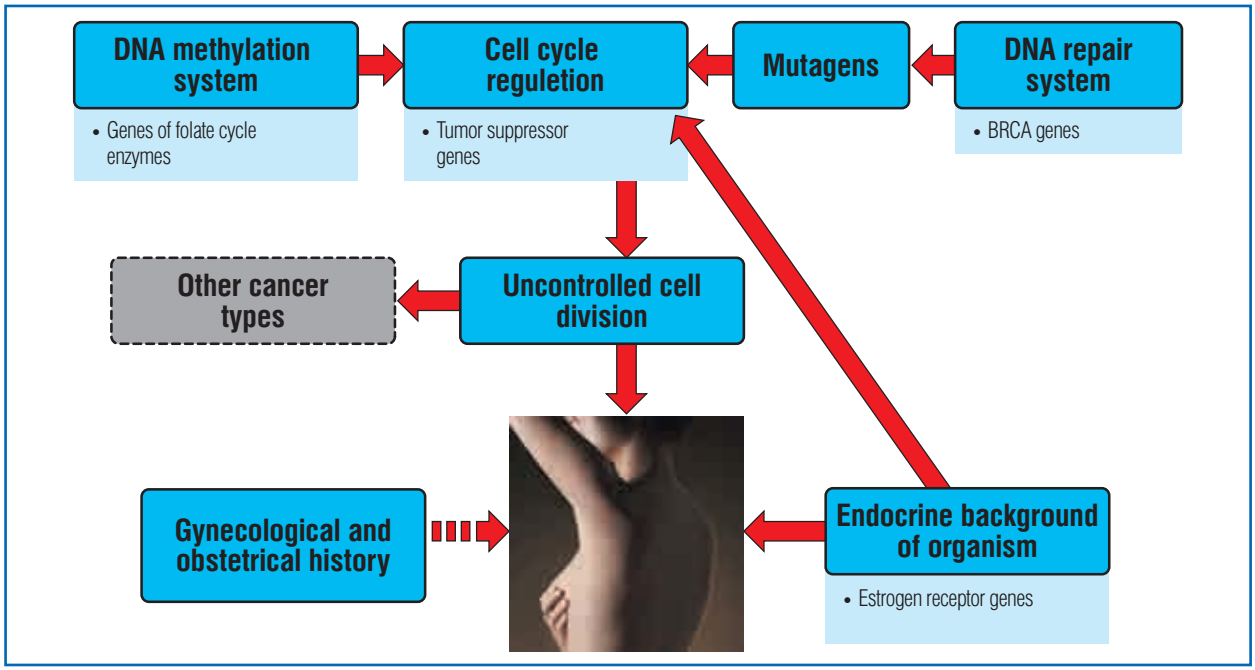


Fig. 27. Gene network. Oncogenetics

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.

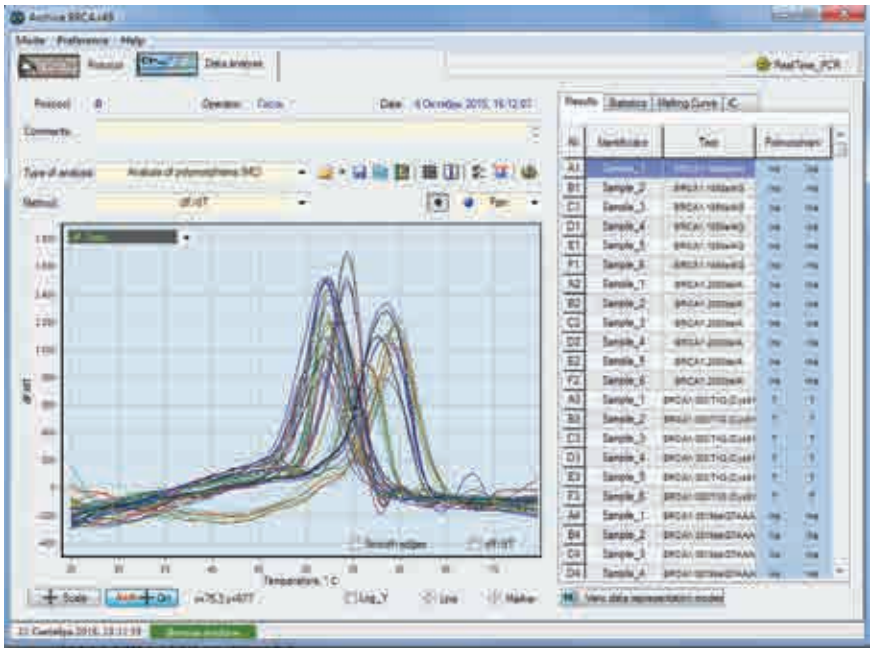
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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

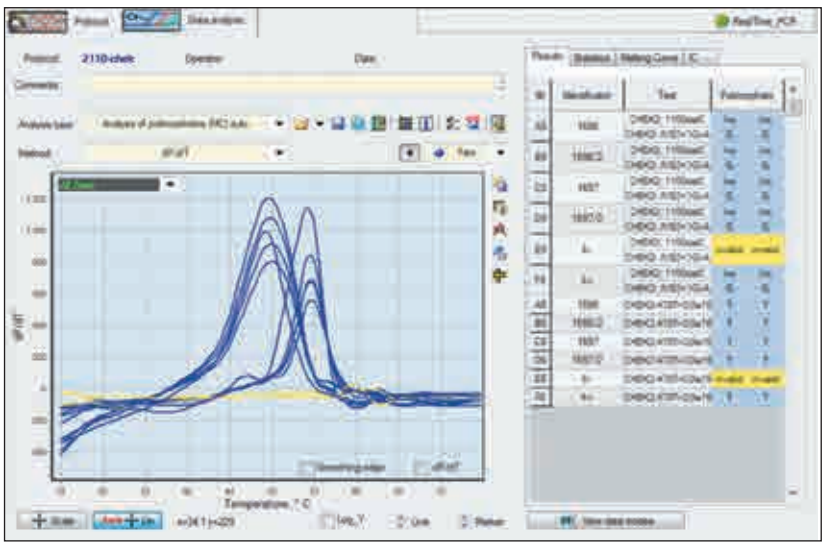
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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.

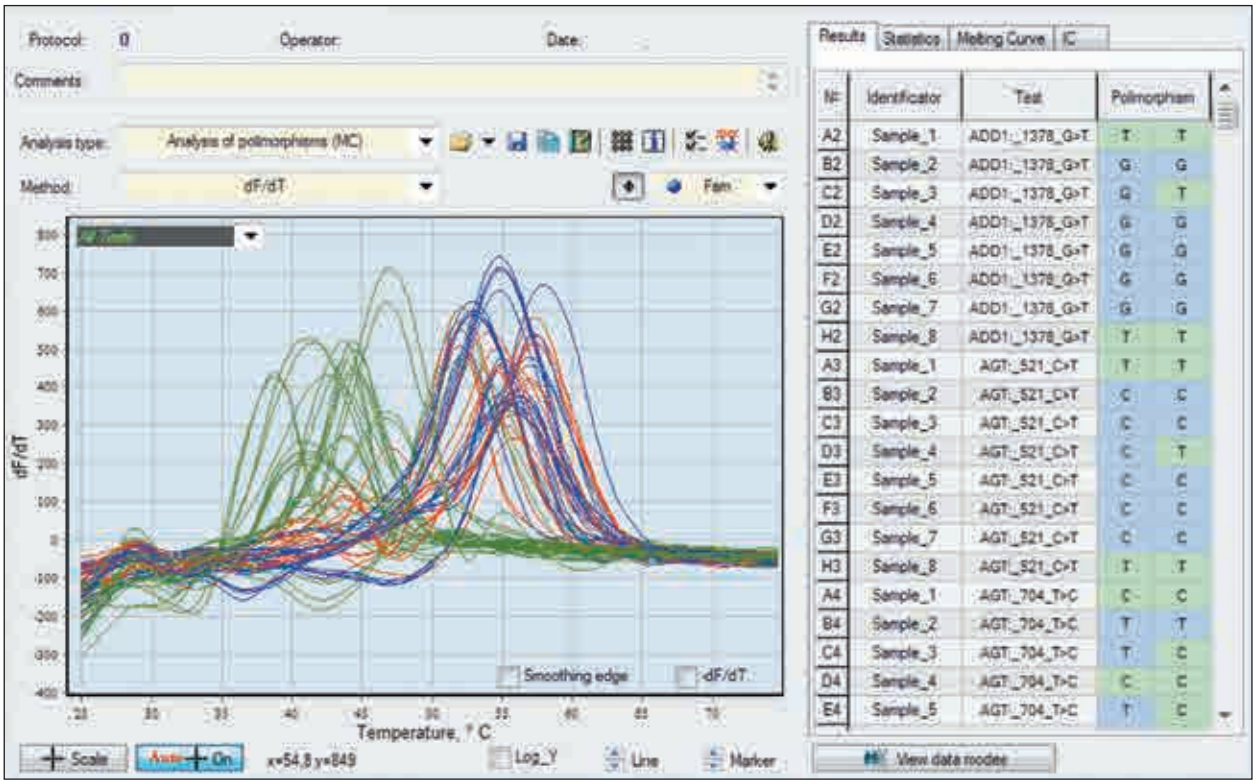
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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.

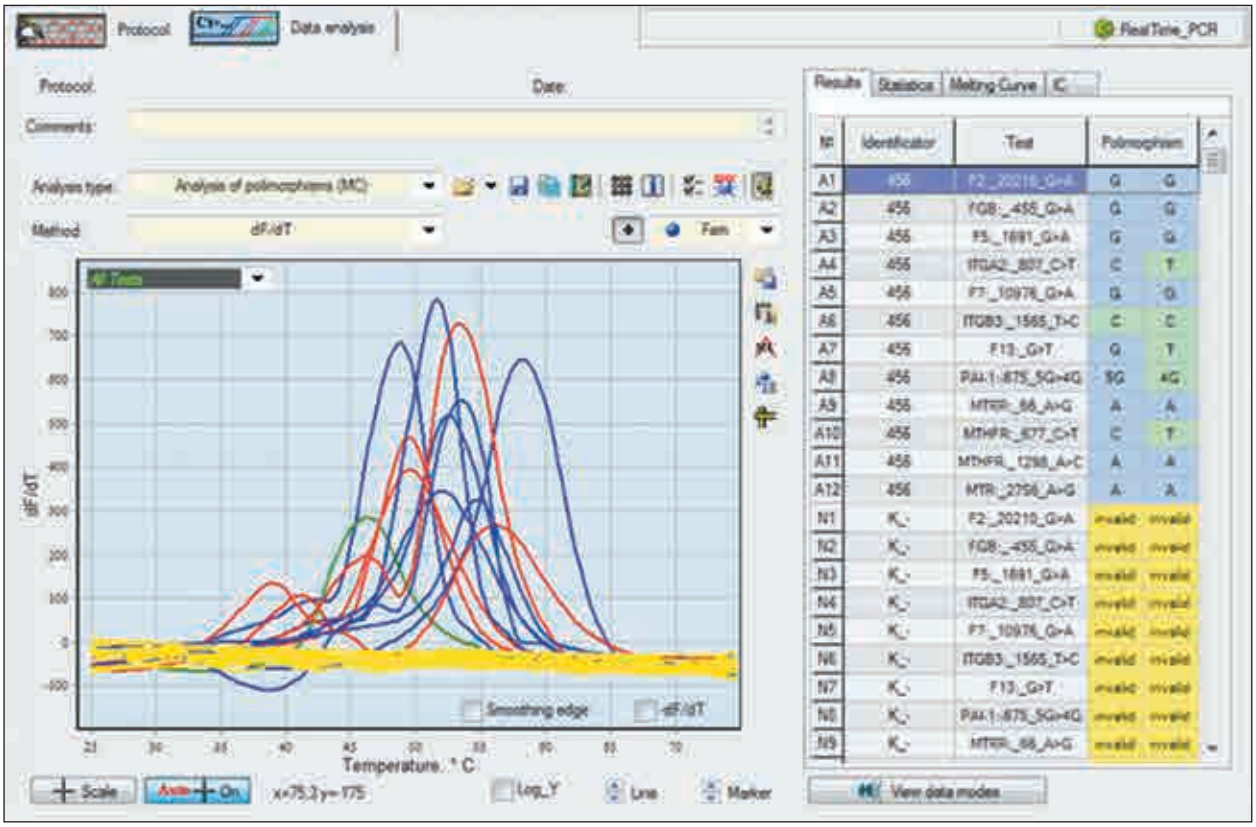
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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).

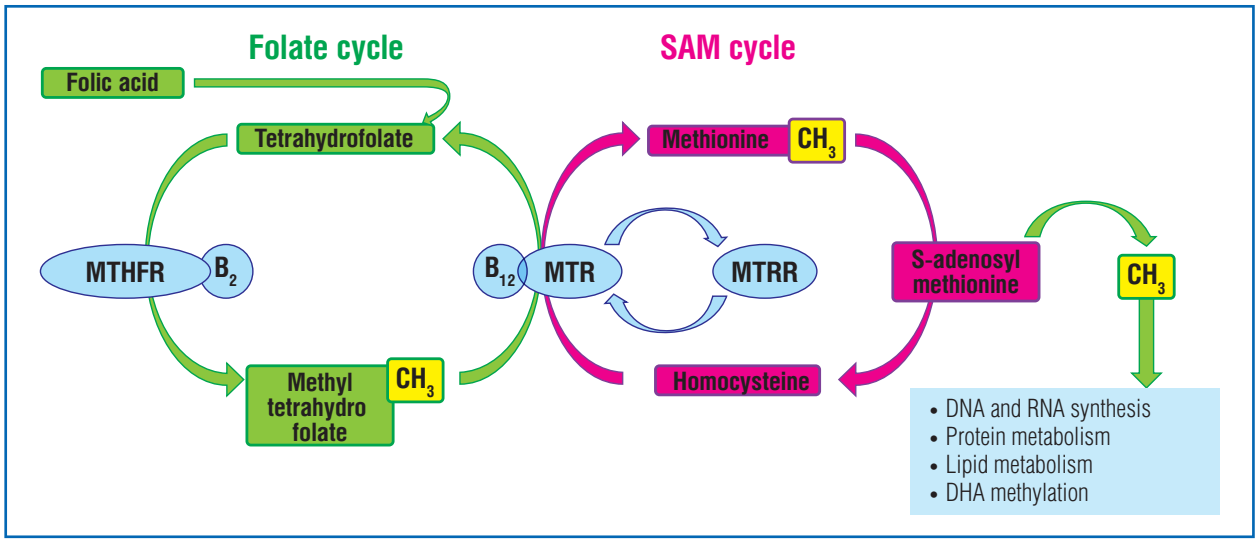


Fig. 32. Folate cycle

Folate cycle is a cascade process controlled by enzymes, which, as coenzymes, have folic acid derivatives. The key step in this process is methionine synthesis from homocysteine. This is achieved in the folate conversion process: 5,10-methylenetetrahydrofolate recovery to 5-methyltetrahydrofolate, carrying a methyl group, which is necessary for homocysteine conversion to methionine. Folate restoration occurs with involvement of methylenetetrahydrofolate reductase (MTHFR). The methyl group is transferred to B12, which then gives it to homocysteine, forming methionine with the help of methionine synthase (MTR). However, in some cases, B12 can be oxidized, resulting in suppression of methionine synthase. To maintain the activity of the enzyme, reductive methylation is necessary, using methionine synthase reductase (MTRR).

Folate cycle disorder leads to accumulation of homocysteine in the cells and increase in the overall homocysteine levels in the blood plasma.

The main form of folate in the plasma is 5-methyltetrahydrofolate, carrying a methyl group, which is necessary for homocysteine conversion to methionine. Because cobalamin (vitamin B12) serves as an acceptor of the methyl group from 5methyltetrahydrofolate, cobalamin deficiency can leads to “folate trapping”, where folates are metabolically dead because they cannot be recycled as tetrahydrofolate back into the folate pool. This leads to depletion of methionine in the blood and release of excess homocysteine, which has an atherogenic effect, hypertensive properties, and increases platelet hyperaggregation [145].

Besides, homocysteine passes freely through the placenta and gives teratogenic and fetotoxic effects [138].

Folate metabolism disorder and elevated homocysteine levels cause increased risk of pathological processes:

- *Pregnancy complications* (fetoplacental insufficiency, preeclampsia, premature detachment of normally situated placenta (PONRP), missed abortion, intrauterine fetal death);
- *Fetal malformations* (defective closure of the neural tube, (spina Bifida), anencephaly, deformation of the facial skeleton), Down syndrome, heart disease) [60, 95, 138];
- *Cardiovascular diseases* (ischemic heart disease, myocardial infarction, atherosclerosis, atherothrombosis);
- *Carcinogenesis* (colorectal adenoma, breast cancer, ovarian cancer, acute lymphocytic leukemia in adults);
- *Increased side effects of chemotherapy*.

Folate cycle disorders do not have an isolated effect on development of venous thrombosis in hormone replacement therapy and oral contraceptives. However, the presence of other thrombophilic polymorphisms (especially factor V Leiden mutation and prothrombin mutation: 20201 G>A) strengthens their effect multiple times [11, 36].

Causes of folate cycle disorders:

- Genetic defects in folate cycle enzymes MTHFR, MTR and MTRR;
- Folic acid deficiency;
- Vitamins B6 and B12 deficiency.

At the same time, it is important to take into account the population features of prevalence of significant polymorphisms associated with defects in folate cycle enzymes. First of all, this concerns the MTHFR gene polymorphism 677 C>T – the most significant in terms of risk of the above pathological states (see Table 56) (Wicken B., 2003).

Table 56. Prevalence of MTHFR 677 C>T allelic variant in different ethnic groups

Territory		Genotype (%)			Allelic variant T (%)
		CC	CT	TT	
Europe	Italy	29.0-33.0	39.0-51.0	15.3-26.4	41.0-46.0
	Spain	44.0-47.0	39.0-44.0	11.8-13.7	33.3-33.9
	France	40.0	48.0	11.8	35.7
	Netherlands	52.0	42.0	6.4	27.4
	Finland	54.0	42.0	4.0	25.1
	Hungary	44.0	45.0	11.1	33.7
	Russia	53.0	40.0	7.0	26.9
Middle East	Israel	57.0	34.0	8.6	25.7
China	North	31.0	49.0	19.8	44.2
	Southern	39.0	53.0	8.1	34.7
Australia		51.0	41.0	8.0	28,6
America	Mexico, USA	18.0	50.0	32.2	57.0
	White population in USA	47.0	42.0	10.7	31.7
	African Americans, USA	78.0	20.0	2.7	12.6
	Latinos, USA	35.0	47.0	17.7	41.1
	Asian population, USA	62.0	35.0	3.8	21.2
	Canada, white population	57.0	38.0	5.8	24.6

Analysis of polymorphisms in folate cycle genes enables to determine predisposition to the above-mentioned pathological processes and allows for timely initiation of a corrective therapy.

Indications for genetic analysis:

- Indications for genetic analysis:
- Elevated homocysteine blood levels (hyperhomocysteinemia);
- Miscarriage, fetal death;
- Birth of a child with isolated defects of the neural tube, heart or urogenital tract;
- Planned preparation for pregnancy;
- Presence of IHD, hypertension, atherosclerosis or atherothrombosis;
- Thromboembolism;
- Antiphospholipid syndrome;
- Family history of cancer;
- Prescription of oral contraceptives and hormone replacement therapy;
- Chemotherapy.

DNA-Technology developed a kit (see Tables 57 and 58) for identification of polymorphisms associated with folate cycle disorders using real-time PCR technique.

Table 57. Genetic polymorphisms associated with folate cycle disorders

Gene	Function of gene product	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
MTHFR – Methylenetetra-hydrofolate	Restores 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (reaction catalyst for formation of 5-methyltetrahydrofolate required for conversion of homocysteine to methionine)	**677 C>T (A222V)**	**rs1801133**	**C/C**	No abnormalities
				C/T	Reduction of functional enzyme activity to 65 % of the average value. Elevated plasma homocysteine levels
				T/T	Reduction of functional enzyme activity to 35% of the average value. Elevated plasma homocysteine levels. In homozygotes, this increase is pronounced much more than in heterozygotes
		1298 A>C (E429A)	**rs1801131**	**A/A**	No abnormalities
				A/C	Combination of heterozygosity of 677T and 1298C alleles is accompanied not only by a decrease in enzyme activity, but also by an increase in plasma homocysteine levels and reduced folate levels
				C/C	Reduced MTHFR activity to about 60 % of normal. Can cause lower plasma folate levels in pregnancy
MTR – B12-dependent methionine synthase	The enzyme is directly involved in homocysteine methylation (reverse conversion of homocysteine to methionine)	**2756 A>G (D919G)**	**rs 1805087**	**A/A**	No abnormalities
				A/G	Homocysteinemia, reduced plasma homocysteine levels in response to increased dietary folates
				G/G	Homocysteinemia, reduced plasma homocysteine levels in response to increased dietary folates
MTRR – Methionine synthase reductase	The enzyme is needed to maintain the activity of methionine synthase by reductive methylation	**66 A>G (I22M)**	**rs 1801394**	**A/A**	No abnormalities
				A/G	Reduced functional enzyme activity, homocysteinemia, especially in combination with 2756 A>G
				G/G	Reduced functional enzyme activity, homocysteinemia, especially in combination with 2756 A>G

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

Table 58. Folate Metabolism Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (96 mcl)
PCR buffer	2 tubes (960 mcl each)
Mineral oil	1 vial (3.84 ml)
Polymorphisms to be identified	MTHFR: 677 C>T – 960 mcl MTHFR: 1298 A>C – 960 mcl MTR: 2756 A>G – 960 mcl MTRR: 66 A>G – 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.

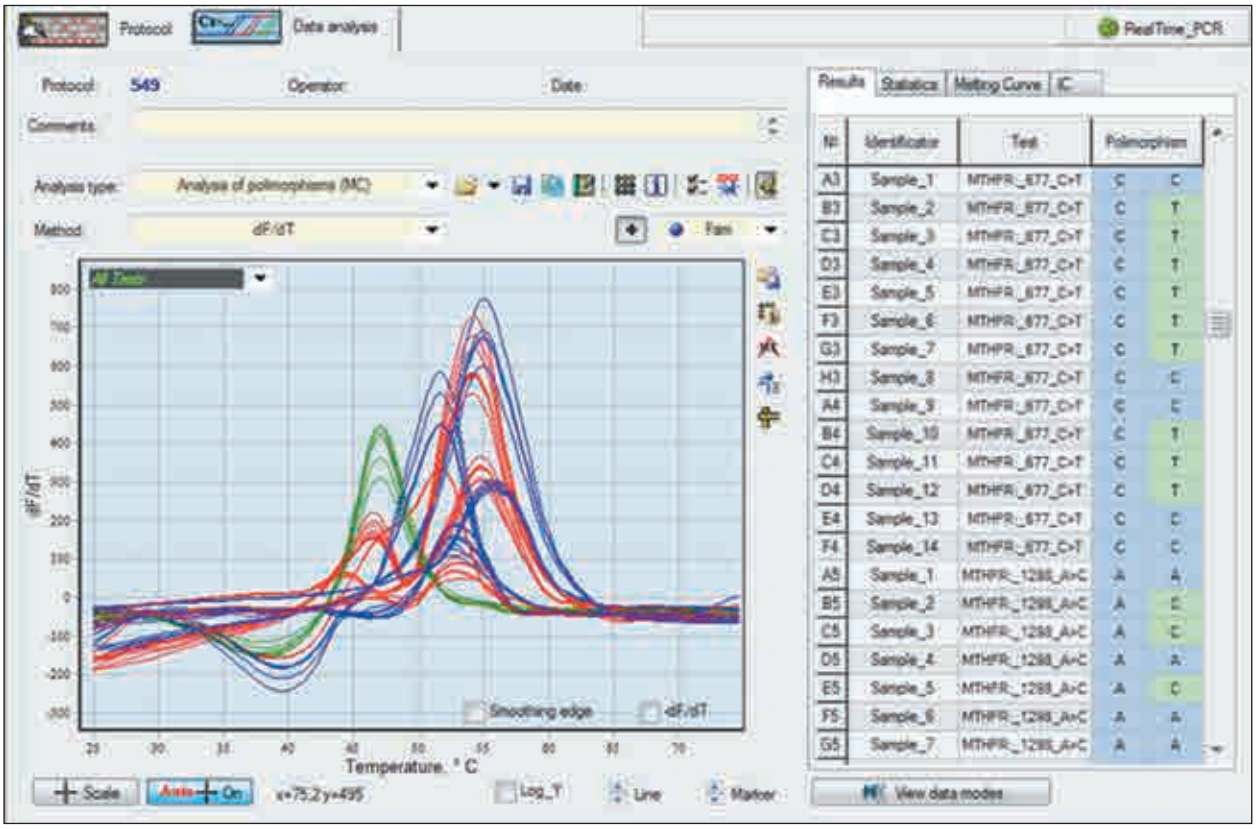
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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. 33).

A



B

№	Name of research	Results	
		Genotype	
1	MTHFR:_677_C>T	C	T
2	MTHFR:_1298_A>C	A	C
3	MTR:_2756_A>G	A	A
4	MTRR:_66_A>G	A	A

Fig. 33. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4.5. Lactose Intolerance Real-Time PCR Genotyping Kit

Lactose is a sugar found in milk, constituting about 99 % of all the carbohydrates in milk. Under the influence of lactase in the digestive tract, lactose splits into glucose and galactose, this occurs after these monosaccharides are absorbed. If there is too much undigested lactose in the intestine (activity of the splitting lactase is reduced), and there is sufficient lactic acid bacteria, then the undigested lactose tigers water flow from the body into the bowel cavity, resulting in loose stools (diarrhea), pain, rumbling and bloating. This condition is called *lactase deficiency* or *lactose intolerance*.

According to severity, LI is divided into:

- *Partial* lactose intolerance;
- Full lactose intolerance.

By origin, there are two main types of lactose intolerance:

- *Primary* lactose intolerance. It is divided into:
  - Congenital (genetically determined, family);
  - Transient (common in premature babies);
  - Adult type.
- *Secondary* lactose intolerance is caused by intestinal infections, as well as by any gastrointestinal tract diseases.

Milk sugar digestion process is associated with activity of lactase-phlorizin hydrolase (lactase or beta-D-galactoside hydrolase). Lactase-phlorizin hydrolase is encoded by a single genetic locus on chromosome 2. Occurrence of primary congenital lactose malabsorption (alactasia in newborns) and primary congenital lactose malabsorption with late onset (in adults) is associated with genetically determined impaired lactase synthesis. Congenital decrease in lactase activity – hypolactasia – is inherited in an autosomal recessive mechanism. The final lactase phenotype is formed in a wide age range: from 5-6 years in Japanese to 20-21 years in the Finns [100, 114].

It has been proven that the MCM6 gene region is one of the most important regulatory elements of lactase gene. It has also been established that polymorphic locus -13910 T>C is linked to lactose intolerance [3, 40, 96, 136].

Indications for genetic analysis:

- Examination of infants with suspected congenital lactase intolerance;
- Differential diagnosis of lactose intolerance and other gastrointestinal disease in children older than one year and in adults.

DNA-Technology developed a kit (see Tables 59 and 60) for identification of polymorphisms associated with lactose metabolism disorders using real-time PCR method.

Table 59. Genetic polymorphisms associated with lactose metabolism disorder

Gene	Function	Polymorphism	Identifier*	Possible genotypes	Effect of polymorphism
MCM6 lactase-phlorizin hydrolase	Regulates LCT gene expression (lactase gene), thereby participating in lactose hydrolysis process	**-13910 T>C**	**rs 4988235**	**T/T**	No abnormalities
				T/C	Reduced MCM6 gene expression. Increases the risk of secondary lactase deficiency
				C/C	Reduced MCM6 gene expression leads to lactose intolerance in adults

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

Table 60. Lactose Intolerance Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (48 mcl)
PCR buffer	1 tube (480 mcl each)
Mineral oil	1 tube (960 mcl)
Polymorphisms to be identified	MCM6: -13910 T>C – 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.

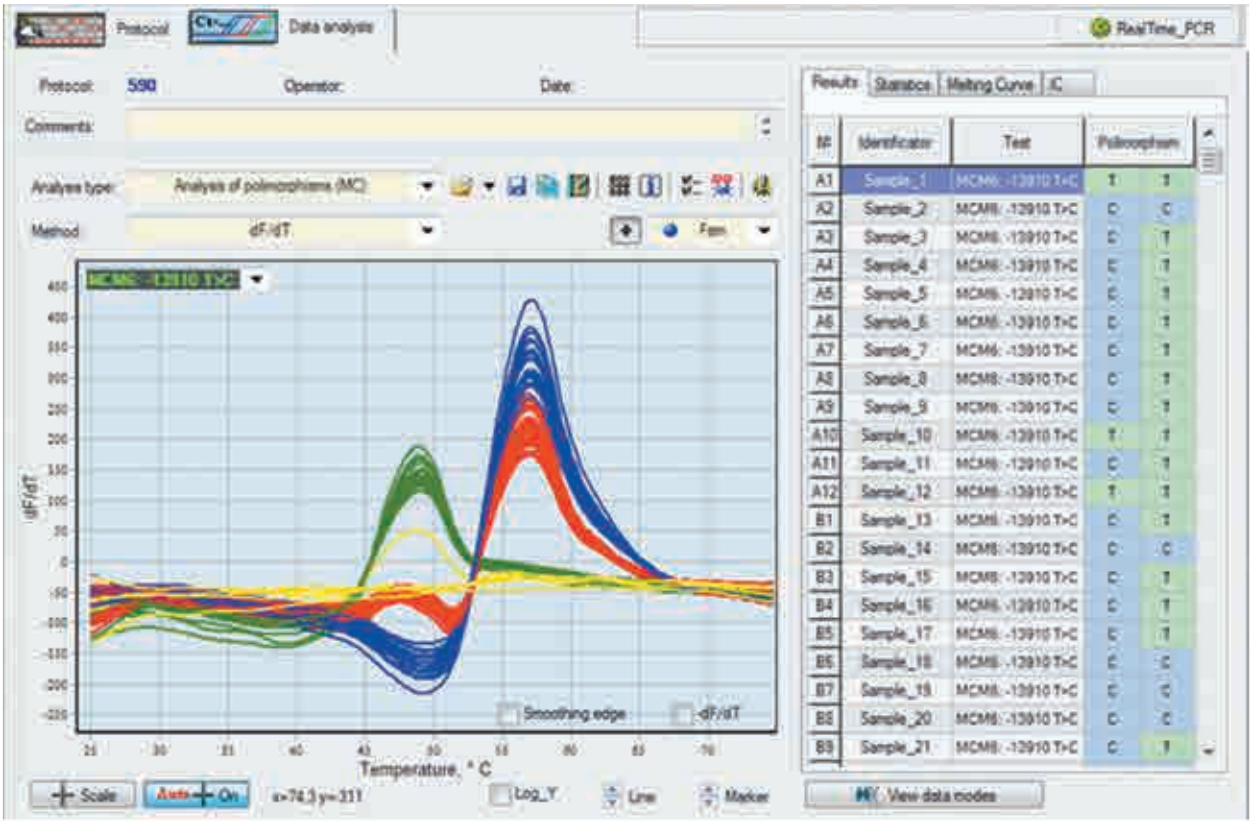
The following equipment and supplies are required for the analysis:

- 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. 34).

A



B

№	Name of research	Results	
		Genotype	Cp
1	MCM6:_-13910_T>C	T T	29,5

Fig. 34. Optical measurement analysis results

A – Optical measurement analysis (Fam channel)

B – Analysis report

12.4.6. Pharmacogenetics

Pharmacogenetics is a section of medical genetics and pharmacology that studies genetically caused individual responses to drugs.

The basic concept of pharmacogenetics consists of various human sensitivity to therapy and effect of certain drugs, depending on genetic characteristics (polymorphic variants of the genotype). The polymorphic nature of many enzymes responsible for their penetration and removal from cells that participate in the metabolism of drugs, and transporters has been established.

Identification of the enzyme activity by genetic typing is one of the simplest and most effective pharmacogenetic approaches to dose correction and pharmacotherapy optimization.

Identification of specific metabolism features plays an important role in the intolerance of certain foods (e.g., milk) and in prescription of certain medications (oral contraceptives and hormone replacement therapy).

The connection between the individual reaction of a person to drug and the genetic defect of the enzyme was f established the first time for succinylcholine, a muscle relaxant. Later, similar studies were carried out for the drugs primaquine and isoniazid. Especially rapid development of pharmacogenetics was achieved after the introduction on the market of the drug warfarin (*Wiskonsin Alumni Research Foundation*) in the market, which showed high efficacy

for prevention of thromboembolic complications in patients with myocardial infarction. The frequency of bleeding complications in patients receiving the drug reached 10-16 %, but an attempt to remove it from clinical practice led to significant increase in post-infarction complications. Determining the genetic causes of individual sensitivity to drug allowed to adjust the dose, strengthen therapy efficacy and avoid complications [49, 64, 65].

For every doctor, identifying the genetic characteristics of a patient allows for personalized approach to choosing the appropriate therapy, determining the most effective dose and reducing side effects. Taken together, this reduces labor losses caused by diseases, lowers involvement of hospital fund and reduces expenses on drugs.

12.4.6.1. Warfarin Pharmacogenetics Real-Time PCR Genotyping Kit

Anticoagulant is a drug that suppresses blood coagulation activity and prevents formation of blood clots.
Types of anticoagulants:

- *Anticoagulants of direct action:* they reduce thrombin activity in the blood (e.g., heparin, hirudin, etc.);
- *Anticoagulants of indirect action:* they are derivatives of oxycumarine and indandione that competitively inhibit vitamin K reductase, which inhibit activation of the latter in the body and stoppage of synthesis of vitamin K-dependent clotting factors (II, VII, IX, X).

Warfarin, an anticoagulant of indirect action, is world's most widely prescribed drug. This anticoagulant was synthesized back in the 40's of the last century. Warfarin owes its popularity to, first, high efficacy, and secondly, ease of use.

Indications for warfarin:

- Treatment and prevention of thrombosis and venous thromboembolism:
  - Acute venous thrombosis;
  - Pulmonary thromboembolism;
- Postoperative thrombosis;
- Recurrent myocardial infarction;
- As an additional drug during surgical or thrombolytic treatment of thrombosis, as well as for electrical cardioversion of atrial fibrillation;
- Recurrent venous thrombosis;

- Recurrent pulmonary thromboembolism;
- Prosthetics of heart valves and blood vessels;
- Thrombosis of peripheral, coronary and cerebral arteries;
- Secondary prevention of thrombosis and thromboembolism after myocardial infarction and atrial fibrillation.

Warfarin may cause major complications of treatment, such as bleeding, the most dangerous of which are gastrointestinal bleeding and brain hemorrhage. The reasons for this are: narrow therapeutic range, interaction with food and other drugs, as well as high individual variability in sensitivity to the drug.

To prevent complications and monitor the effect of the drug during treatment, it is necessary to dynamically measure INR (*international normalized ratio*). At the initial stage of treatment, monitoring should be carried out daily.

Despite determining the dosage of indirect-action anticoagulants under INR control, the bleeding frequency remains high, ranging from 10 to 25 %. This shows that genetic factors are paramount in selection of individual warfarin doses, especially the starting doses [34, 44].

It is shown that:

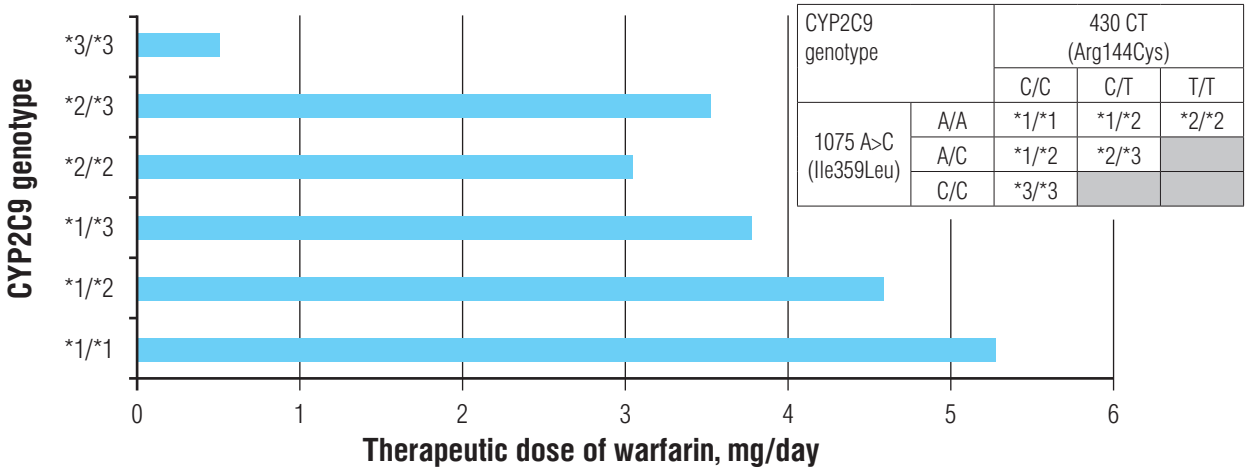
- Genetic factors determine up to 53-54 % of dose variability;
- Clinical factors determine 17-21 % of dose variability.

Currently, the U.S. Food and Drug Administration (FDA), an agency of the U.S. Department of Health and Human Services, has a list of drugs for which the use of genetic tests is seen as desirable, and in some cases, necessary (for example, warfarin). FDA has also approved new labeling for anticoagulant Coumadin, which indicates that the effect of the drug may not be the same for different people because of the individual structure of the genome of each individual. The FDA also made it mandatory for warfarin (generic Coumadin) manufacturers to make similar labeling (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetetics/ucm083378.htm). The most important genes that determine individual response to warfarin therapy are [117]:

- CYP2C9;
- CYP4F2;
- VKORC1.

VKORC1 is a one complex subunit of *vitamin K epoxide* reductase – a key enzyme of vitamin K cycle. Warfarin gives anticoagulant effect through inhibition of VKORC1, which leads to suppression of activation of clotting factors.

Cytochromes **CYP2C9** and **CYP4F2** are enzymes responsible for warfarin metabolism in the body. They participate in metabolism of clinically significant S-warfarin, which is 5 times more potent than R-warfarin. CYP2C9 and CYP4F2 polymorphisms are associated with changes in functional activity of the enzymes, which affects the rate of warfarin removal from the body and, therefore, affects individual sensitivity to anticoagulant therapy (fig. 35).



CYP2C9 genotype		430 CT (Arg144Cys)		
		C/C	C/T	T/T
1075 A>C (Ile359Leu)	A/A	*1/*1	*1/*2	*2/*2
	A/C	*1/*2	*2/*3	
	C/C	*3/*3		

Fig. 35. Different warfarin sensitivity, depending on genotype
(source: Lee CR, Goldstein JA, Pieper JA. Cytochrome P450 2C9 polymorphisms: a comprehensive review of the in-vitro and human data. Review. Pharmacogenetics 2002;12(3):251–263).

Nevertheless, it is important to take into account population and ethnic sensitivity to the drug and the importance of certain polymorphic variants of genes in this process. The key role is played by the VKORC1 gene polymorphism, since it accounts for up to 30 % of dose variation in Europeans and about 10 % in African Americans [81]. The allelic variants of these genes is the least prevalent among Africans and African-Americans (see Table 61).

Table 61. Prevalence of VKORC1 and CYP2C9 alleles in different populations [133].

Population	CYP2C9*1 (wild-type)	CYP2C9*2	CYP2C9*3	VKORC1 C1173T
Europeans	74.3 %	11.3-14.3 %	8.4-10.9 %	42.2 %
Asians	98.4 % (Japan)	0	1.6 %	89.1 % (Japan)
African Americans	95.3 %	0-2.9 %	0.8-1.8 %	8.6 %

In this regard, there are two main haplotypes [112]:

- **Low-dose haplotype group** (<21 mg/week) – Asians and Europeans tend to be more sensitive to the action of warfarin (a faster achievement of INR level, but more frequent bleeding)
- **High-dose haplotype group** (over 49 mg/week) – African Americans are relatively resistant to the action of warfarin

Based on genetic test results, a doctor or clinician can estimate an expected saturated and therapeutic dose of warfarin for each individual patient. The site http://www.warfarindosing.org contains a program that allows you to calculate the dose, taking into account clinical and genetic factors (Fig. 36).

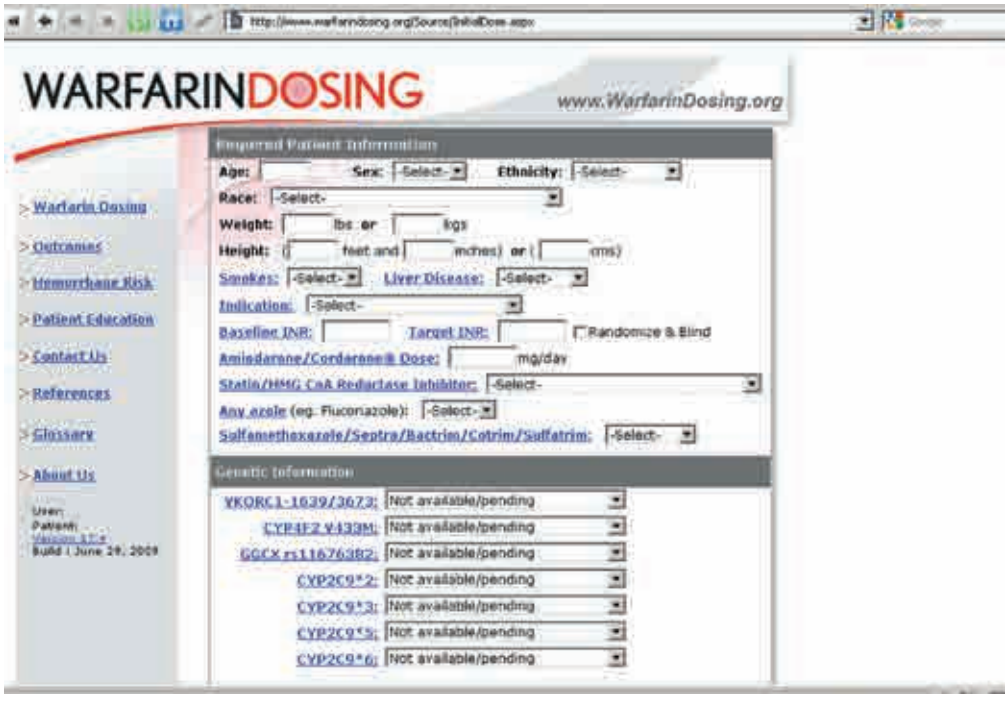


Fig. 36. Calculator for calculating warfarin doses, taking into account clinical and genetic factors

A randomized, prospective study *WARFAGEN* was organized under the auspices of the Russian Society of Cardiology (RSC), the All-Russian Scientific Society of Cardiology (ARSSC) and the Atherothrombosis National Society (ANS), to implement a pharmacogenetic approach to selection of warfarin dose in the Russian population. It was found that among Russian patients in need of warfarin, the total frequency of genotypes that determine clinically significant disorder in warfarin sensitivity is 31.5 %.

DNA-Technology developed a kit of reagents (see Tables 62 and 63) for detecting polymorphisms associated with warfarin metabolism using real-time PCR technique.

Table 62. Genetic polymorphisms associated with warfarin pharmacogenetics

Gene	Gene function	Polymorphism	Identifier*	Possible genotypes	Association / Effects
CYP2C9 – cytochrome P450	Encodes an enzyme involved in S-warfarin metabolism CYP2C9*1 genotype (wild-type) – causes normal metabolism of the drug	**CYP2C9*2 430 C>T (Arg144Cys)**	**rs1799853**	**C/C (*1)**	No abnormalities
				C/T (*2)	Reduced functional enzyme activity, increased warfarin metabolism duration
				T/T (*2)	
		1075 A>C (Ile359Leu) CYP2C9*3	**rs1057910**	**A/A (*1)**	No abnormalities
				A/C (*3)	Reduced functional enzyme activity, increased warfarin metabolism duration
				C/C (*3)	
CYP4F2 – leukotriene B4 omega-hydroxylase 1, from the P450 family	Encodes an enzyme involved in vitamin K cycle as a regulator of formation and activation of clotting factors	**1347 C>T (Val433Met)**	**rs2108622**	**C/C**	No abnormalities
				C/T	Reduced functional enzyme activity
				T/T	
VKORC1 – one complex subunit of vitamin K epoxide reductase	Encodes a subunit of an enzyme involved in formation of the active form of vitamin K and subsequent activation of clotting factors	**-1639 G>A**	**rs9923231**	**G/G**	No abnormalities
				G/A	Enzyme deficiency, reduced intensity of formation of the active form of vitamin K
				A/A	

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

Table 63. Warfarin Pharmacogenetics Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (96 mcl)
Mineral oil	1 vial (3,84 ml)
PCR buffer	2 tubes (960 mcl each)
Polymorphisms to be identified	VKORC1: -1639 G>A – 960 mcl CYP2C9: 430 C>T – 960 mcl CYP2C9: 1075 A>C – 960 mcl CYP4F2: 1347 C>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.

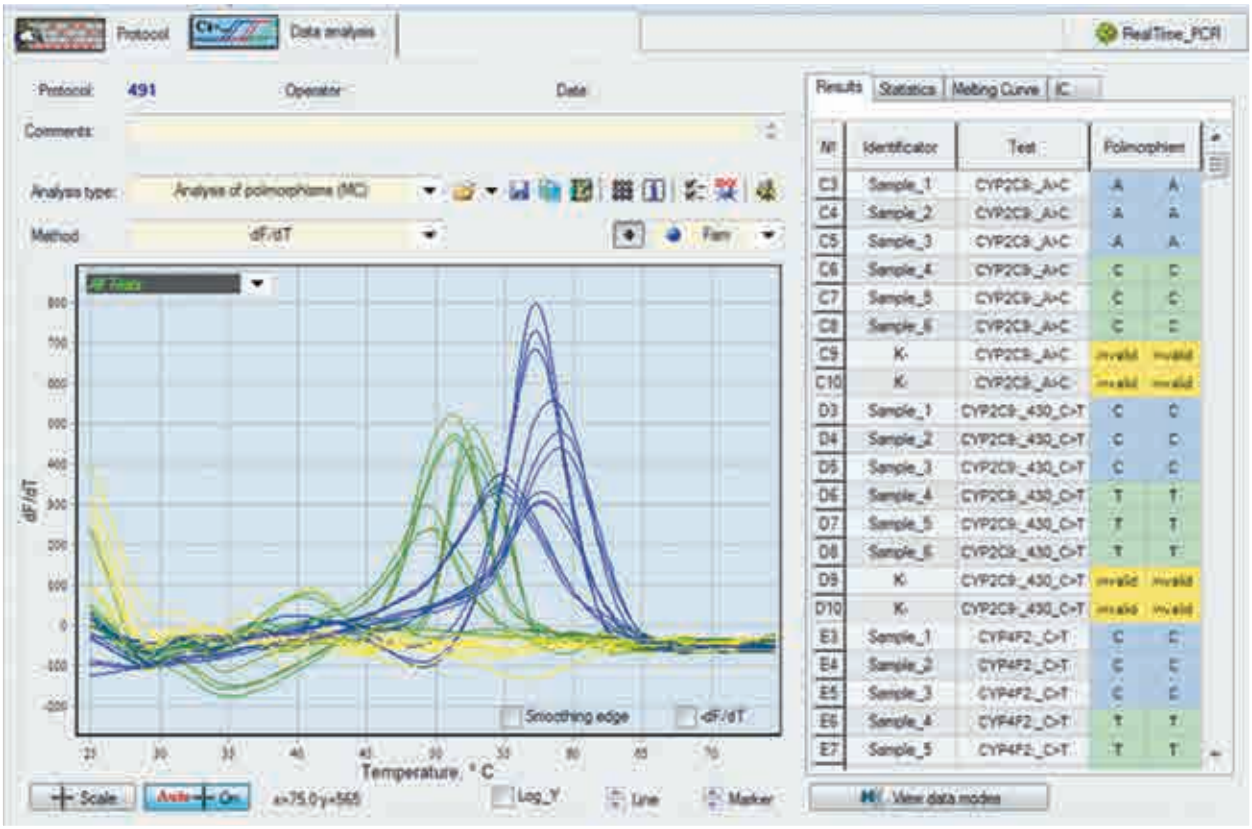
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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. 37).

A



B

№	Name of research	Results
		Genotype
1	CYP2C9:_A>C	A S
2	CYP2C9:_430_C>T	C C
3	CYP4F2:_C>T	C C
4	VKORC1:_-1639_G>A	G G

Fig. 37. Optical measurement analysis results
A – Optical measurement analysis (Hex channel)
B – Analysis report

12.4.6.2. Clopidogrel Pharmacogenetics Real-Time PCR Genotyping Kit

Antiplatelet drugs are a mandatory component for secondary prevention of thrombotic and thromboembolic complications. Major meta-analysis *Antithrombotic Trialists' Collaboration*, bringing together the results of 145 clinical studies, showed that the use of antiplatelet therapy in patients at high risk reduces the risk of cardiovascular complications by 25 %.

Especially significant benefits of antiplatelet therapy have been reported in patients with acute coronary syndrome (ACS), as well as those who underwent percutaneous intracoronary intervention (PII) in the coronary arteries, primarily with placement of intracoronary stents.

Today, it has been proven that for many categories of cardiovascular high-risk patients, prolonged antiplatelet therapy is preferred in the form of combination of two drugs with different mechanisms of action. To date, the most compelling evidence is from a combination of acetylsalicylic acid (ASA) and clopidogrel.

Clopidogrel is one of the world's currently most widely used antiplatelet agent, which irreversibly inhibits platelet ADP receptor (encoded by P2RY12), which in turn inhibits fibrinogen receptor (GP IIb/IIIa, encoded by ITGB3). Unlike ASA, thienopyridines do not alter arachidonic acid exchange and, therefore, do not disturb formation of prostacyclin in the vascular wall.

Clopidogrel pharmacogenetics

The action of clopidogrel on the receptor is irreversible and, accordingly, the consequent suppression of platelet agent is maintained for the remaining life of the plates.

The drug is a prodrug and needs complex transformation to CYP450 in order to give specific antiplatelet effect. CYP2C19 is the main enzyme behind clopidogrel metabolism. Only 15 % of administered clopidogrel is converted into active substance acting on ADP receptor [1]. At the same time, the main clopidogrel metabolite, which is available for definition in pharmacogenetics studies, does not have an antiplatelet effect.

Features of clinical use of clopidogrel

There is considerable inter-individual variability in pharmacodynamic response when using clopidogrel. Patients with insufficient platelet response to clopidogrel may have an increased risk of ischemic events. About 8-10 % of patients with acute coronary syndrome undergoing modern treatment suffer recurrent cardiac events in the first year. Besides, 1-3 % of patients subjected to PII develop acute or subacute stent thrombosis, which can have catastrophic consequences, including death. "Resistance" to clopidogrel is often mentioned among the explanations for the persistent risk of thrombotic / ischemic events [2].

Clopidogrel stability (resistance), which is observed in a significant proportion of patients (25 %) with acute myocardial infarction with ST segment elevations, is associated with increased risk of recurrent cardiovascular events [2]. Moreover, none of the functional tests for resistance detection is still recommended for application in routine clinical practice [3].

Pharmacogenetic approach

The **CYP2C19 gene** has pronounced polymorphism, which may lead to a change or total loss of enzymatic activity of the protein. It is shown that the presence of alleles associated with reduced gene activity manifests phenotypically in the form of insufficient enzyme activity (poor metabolizers). Genotyping is recommended to detect allelic variants of the P450 cytochrome, associated with changes in the enzyme activity (level of evidence C) [86, 120].

Allele CYP2C19*1 corresponds to a fully functional enzyme, whereas alleles CYP2C19*2 and CYP2C19*3 cause reduced metabolism. They account for 85 % of all alleles that reduce the function of the enzyme in European and 99 % in Asians. Other alleles associated with reduced metabolism include CYP2C19*4, *5, *6, *7, and *8, but are extremely rare [144].

In the study titled *Thrombolysis in Myocardial Infarction* (TIMI), it was shown that among the patients who received clopidogrel and were carriers of functionally impaired CYP2C19 allele, more than 50% had an increased risk of death from heart attack or stroke as a primary outcome compared to non-carriers. It was proven that the risk of getting stent thrombosis with this carriage was three times higher [126]

Results from the *French Registry of Acute ST-Segment Elevation and Non-ST-elevation Myocardial Infarction* (FAST-MI) have shown that patients carrying any of the CYP2C19 reduced-function alleles, which include CYP2C19*2, CYP2C19*3, CYP2C19*4 or CYP2C19*5, have a significantly higher risk of death from myocardial infarction or stroke [91]

According to experts from the *American Heart Association and American College of Cardiology,* ***it is advisable to perform preliminary genotyping in patients undergoing high-risk planned procedures*** (for example, in complex coronary anatomy or multivascular damage). In identifying low-activity risk alleles of the CYP2C19 gene, such patients should be given other P2Y12 receptor blockers or use higher clopidogrel doses [57].

In 2009, new information has been added to the prescribing label for clopidogrel concerning pharmacogenetics: "*Pharmacogenetic testing can identify genotypes associated with variability in CYP2C19 activity*".

*17 allele is associated with increased gene transcription. Therefore, homozygous carriers of this allele belong to the highly active metabolizers. Presence of *17 allele increases the risk of adverse drug reactions when taking standard doses of prodrugs that are activated through CYP2C19, which may require dose reduction [101].

The **ABCB1 gene** – ATP-binding cassette, sub-family B (**MDR1**) encodes transporter protein which is involved in removal of drugs from the cells, including clopidogrel and other xenobiotics. Substitution at position 3435 C>T is synonymous and does not alter the amino acid sequence of the protein, (Ile1145Ile), but changes its substrate specificity [69, 78].

T allele carriers have an increased risk of adverse cardiovascular events (cardiac death, myocardial infarction) in treatment of acute coronary syndrome or heart attack with clopidogrel (evidence level 3 **http:// PharmGKB.org**).

DNA-Technology developed a kit (see Tables 64 and 65) for identification of genetic polymorphisms associated with clopidogrel metabolism using real-time PCR method.

Table 64. Genetic polymorphisms associated with Clopidogrel pharmacogenetics

Gene	Gene function	Polymorphism	Identifier*	Possible genotypes	Association / Effects
CYP2C19 enzyme – cytochrome P450, subfamily IIC	Encodes hormones (e.g., estrogen and progesterone) activation or metabolism factors and a number of drugs, including anti-epileptic medications, anti-depressants, antiplatelet agents, proton pump inhibitors and certain antimalarials.	**CYP2C19*1**	**n/a**		No abnormalities
					Causes normal/fast drug metabolism
		CYP2C19*2 681 G>A	**rs4244285**	**GG**	No abnormalities
				GA	Weak drug metabolism. Risk of low efficacy of antiplatelet therapy (clopidogrel) and high probability of cardiovascular complications
				AA	
		CYP2C19*3 636 G>A	**rs 4986893 или rs57081121**	**GG**	No abnormalities
				GA	Weak drug metabolism.
				AA	
		CYP2C19*17 -806 C>T	**rs12248560**	**CC**	No abnormalities
				CT	Accelerated drug metabolism. Possibility of using lower clopidogrel doses in order to avoid complications from standard-dose therapy.
				TT	
AVSV1 – ATP-binding protein, subfamily B	P-glycoprotein of a cellular membrane encoded by the ABCB1 (CD243) gene, a transporter of chemical compounds from the cell.	**3435 C>T**	**rs1045642**	**CC**	No abnormalities
				CT	Reduced bioavailability of clopidogrel. Risk of recurrent cardiovascular events amid therapy
				TT	

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

Table 65. Clopidogrel Pharmacogenetics Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	1 tube (96 mcl)
Mineral oil	1 vial (3,84 ml)
PCR buffer	2 tubes (960 mcl each)
Polymorphisms to be identified	ABCB1: 3435 C>T– 960 mcl CYP2C19: 681 G>A*2 (P227P) – 960 mcl CYP2C19: 636 G>A*3 (W212X) – 960 mcl CYP2C19: -806 C>T *17 – 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.

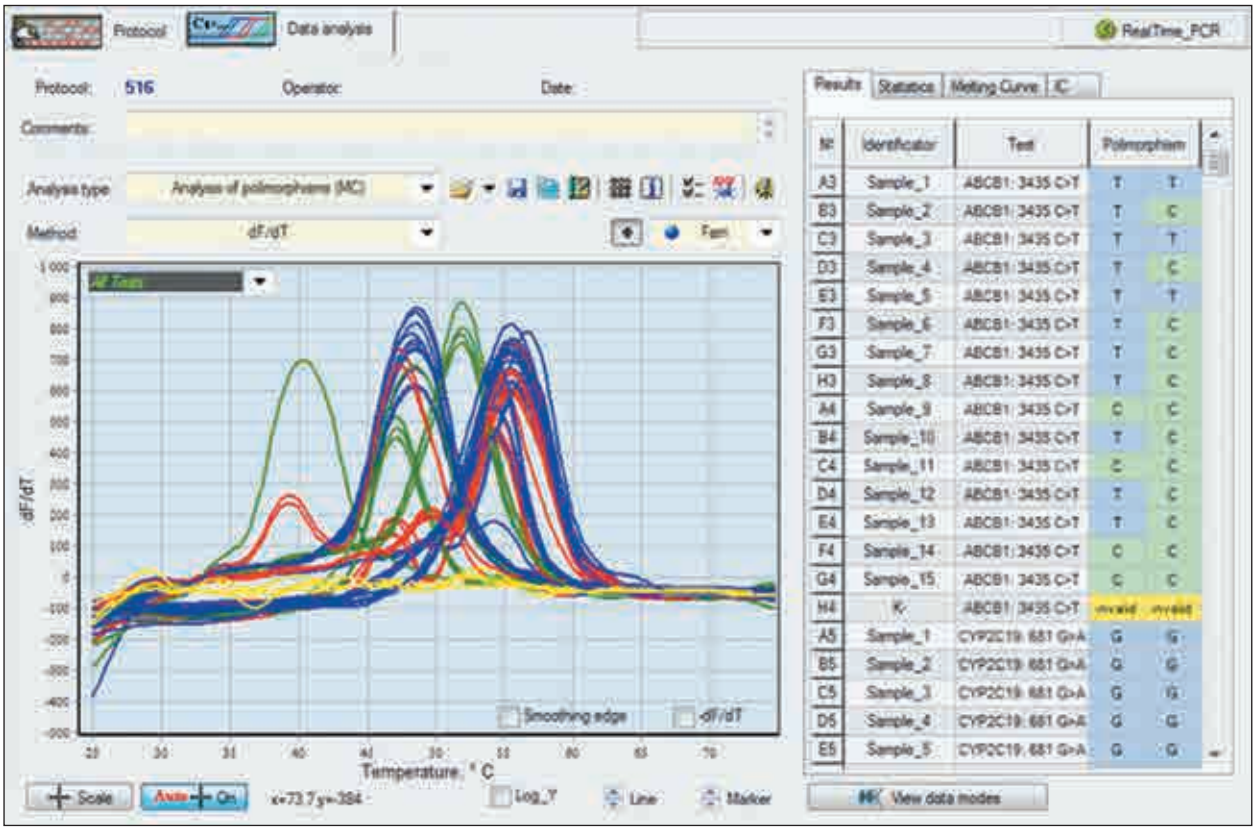
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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. 38).

A



B

№	Name of research	Results	
		Genotype	
1	ABCB1:_3435_C>T	T	T
2	CYP2C19:_681_G>A	G	G
3	CYP2C19:_636_G>A	G	G
4	CYP2C19:_-806_C>T	C	T

Fig. 38. Optical measurement analysis results
A – Optical measurement analysis (Hex channel)
B – Analysis report

12.4.7. Immunogenetics. IL28B Real-Time PCR Genotyping Kit

Hepatitis C virus is a widespread worldwide infectious disease caused by hepatitis C virus (HCV), which has infected about 170 million people [131].

After acute hepatitis C, the virus persists in the blood of 85-90 % of patients, regardless of the infection route. In 50-70 % of patients, the disease subsequently becomes chronic hepatitis. Often, chronic hepatitis progresses very slowly and has no clinical manifestations for a long time. However, even in mild chronic hepatitis C, almost 30 % of patients develop cirrhosis after 30 years, which can cause hepatocellular cancer [5, 42, 103].

In chronic hepatitis C, previously considered an incurable disease, modern antiviral therapy allows to eradicate the virus, prevent development of serious complications of chronic viral hepatitis (cirrhosis, hepatocellular carcinoma), and preserve working capacity in 40-60 % of patients [31].

Over the years, interferon alpha monotherapy has been the basis for treatment of hepatitis C. The modern antiviral treatment involves pegylated interferon plus ribavirin combination therapy (PEG-IFN/RBV) as recommended by the *American Association for the Study of Liver Diseases* (AASLD) in 2009 and the *European Association for the Study of the Liver* (EASL) in 2011 [35].

The goal of treatment is to achieve sustained virological response (SVR), i.e. long (at least 6 months) absence of the detected virus in the blood after termination of antiviral therapy. SVR is accompanied by normalization of biochemical parameters, improvement in liver histology (decrease in histology activity index and fibrosis index) and improvement in the quality of life of patients [48, 52, 116].

The tactics for antiviral therapy for chronic hepatitis C is determined by the rate of virological response, the so-called virological response-guided therapy. Recent years have witnessed emergence of a method used to predict response to therapy based on viral kinetics of early stage of treatment. Duration of treatment also depends on virological response. Virological control by PCR on the 4th, 12th and 24th week of therapy allows to estimate the optimal treatment duration [87, 139].

The following are response options:

- Rapid virologic response (RVR) (aviremia at 4th week of treatment);
- Early virologic response (EVR) (estimated at 12th week of treatment):
  - Complete early virologic response(cEVR) (aviremia);
  - Partial early virologic response (pEVR) (reduction in viral load by more than 2 Log IU/ml);

- Virological non response (VNR) (aviremia at 24th week of treatment);
- Null response (NR) – decreased viral load by by at least 2 Log ME/ml after 12 weeks of therapy.

Duration of treatment required to achieve SVR is determined by the rate of reduction in blood viral load and by baseline characteristics such as fibrosis stage, level of HCV viral load and HCV genotype.

Sustained virological response (SVR) is a reliable criterion for elimination of the virus from the body, which can be viewed as a cure. The probability of SVR is directly proportional to the time of disappearance of HCV RNA during treatment.

The main predictor of therapy success is RVR achievement when using quantitative PCR method with sensitivity of ≤50 IU/ml. Achievement of RVR is a predictor of the onset of SVR regardless of genotype and treatment regimes used [35].

Unfortunately, under prolonged antiviral therapy (48 or 72 weeks in HCV genotype 1), the patient's quality of life may deteriorate and side effects of treatment may appear: neuropsychological disorders (irritability, insomnia, depressed mood, fatigue and malaise), fever, reduction in hemoglobin levels, changes in thyroid function, alopecia and dry skin. Sometimes, the side effects are so pronounced that treatment has to be discontinued.

Obviously, identification of therapy response determinants, including genetic determinants, is of great importance, since doctors need criteria for prediction of treatment efficacy. In other words, before commencement of a standard therapy, the patient must be informed of the link between the likelihood of achieving SVR and the risk of side effects of the antiviral drugs used.

It is now well established that changes in cytokine gene cluster (IL28A, IL28B and IL29), localized on chromosome 19 (19q13) of a person, are the main determinant of anti-viral defense (Fig. 39). Polymorphism in the region adjacent to the interleukin 28B (IL28B) is of greatest importance. Both of these polymorphisms label the portion that is important for response to antiviral therapy.

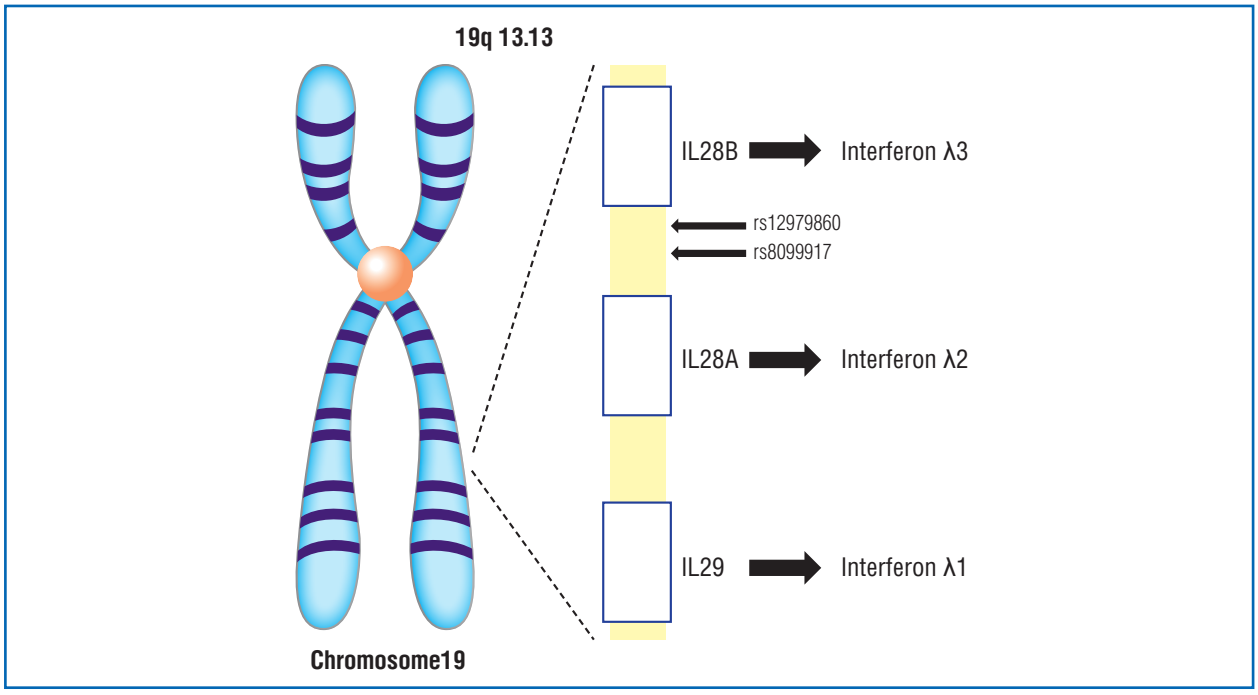


Fig. 39. Localization of the IL28B gene and two functionally significant polymorphisms rs12979860 C>T and rs8099917 T>G

IL28B is interferon-λ-3, and is a ligand of class II cytokine receptor. These ligands trigger JAK/STAT signaling pathway by activating synthesis of 2',5'-oligoadenylate synthase, which activates endonuclease. Endonuclease, in turn, is involved in stimulation of the enzyme protein kinase, which blocks synthesis of viral proteins. It is shown that IL28B polymorphisms are defined both as the probability of spontaneous HCV elimination, and response to interferon plus ribavirin combination therapy [62, 66].

Two single nucleotide substitutions play the major role in hepatitis C virus infection:

- **Cytosine-to-thymine substitution (C>T)**, labeled as **rs12979860** in the dbSNP database of the National Center for Biotechnological Information, NCBI;
- **Thymine-to-guanine substitution (T>G)**, labeled as **rs8099917**.

Carriers of the **rs12979860 CC genotype** have a twofold increase in likelihood of positive response to interferon plus ribavirin combination therapy – both among patients of European ancestry ($p = 1.06*10^{(-25)}$) and in African Americans ($p=2.06*10^{(-3)}$). Furthermore, the efficacy of treatment is actually not directly related to race or ethnicity – African Americans are more often carriers of the T/T genotype than the Europeans and Asians. The CC genotype is mostly found in people with spontaneous infection elimination. Interestingly, for CC genotype, the viral load (amount of virus in the blood) before treatment is higher than for T/T genotype [134].

The T/T genotype is associated with spontaneous infection elimination, regardless of treatment [54].

G allele for **rs8099917** is a risk allele associated with low response to pegylated interferon plus ribavirin combination therapy [90, 111].

Studies on the role of genetic polymorphisms in these regions of the human genome have shown that the positive predictive value of IL28B is higher than other predictors of treatment success (body mass index, age, stage of fibrosis, and viral load) [124].

However, studies on large populations of CHC patients with a different clinical profile (stage of fibrosis from 0 to 4, low and high initial level of viremia), it was shown that the predictive value of IL28 genotype for SVR achievement can significantly be modified by these clinical characteristics and decrease to 37.3 % from 74.4 % for patients with CC genotype [2, 101].

Therefore, it is still necessary to consider the basic characteristics of the disease stage of a specific patient, but in complex with its genetic markers.

There are currently 11 identified genotypes, which in turn are presented in several subtypes. Different genotypes and subtypes are distributed unevenly in different regions of the world. In Russia, the predominant genotype is 1b, followed by 3a, 1a and 2a in a decreasing frequency. It is shown that the IL28B polymorphism has the greatest value at infection by HCV genotype 1 subtype [7, 110].

Based on this, an **screening algorithm** used to **prepare for treatment** is recommended (Fig. 40) .

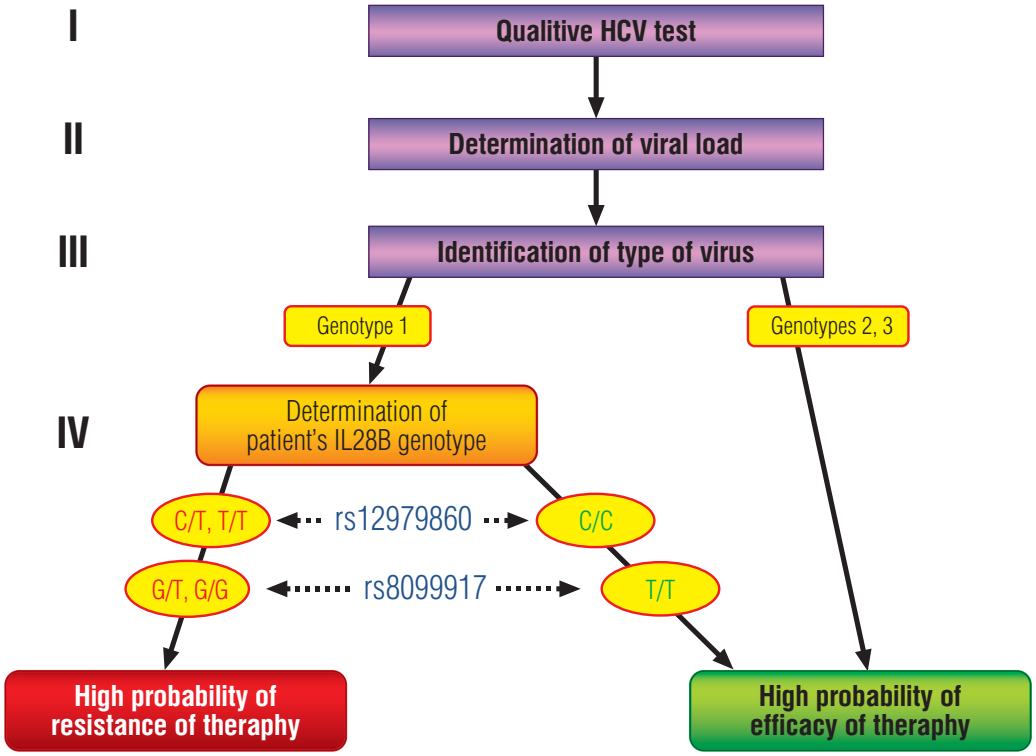


Fig. 40. Recommended screening algorithm before beginning hepatitis C therapy

The Asian population has a strong disbalance in adhesion between rs12979860 and rs8099917, but the European population has a weaker adhesion, which in some cases, requires analysis of haplotypes (see Table 66).

Table 66. Identification of haplotype on combination of genotypes rs12979860 and rs8099917

Генотип		rs 8099917		
		T/T	T/G	G/G
rs 12979860	C/C	CT/CT		
	C/T	CT/TT	CT/TG	
	T/T	TT/TT	TT/TG	TG/TG

The CC rs12979860 genotype has a stronger positive effect on treatment than the TT rs8099917 genotype. On the other hand, TT rs12979860 and GG rs8099917 combination is more common in patients not responding to treatment. The adverse GG rs8099917 genotype turned out to be the most important in predicting the lack of response to treatment [93].

Identification of a patient's genotype on IL28B may change the treatment algorithm (change in the duration of standard therapy PEG-IFN/RBV or triple CHC therapy). Optimization of therapy will help avoid side effects and further additional costs for triple therapy with protease inhibitors (telaprevir and boceprevir).

Indications for genetic analysis:

- Prediction of disease outcome;
- Choice between standard and pegylated interferon plus ribavirin combination therapy for CHC treatment;
- Choice of the tactics "waiting for accessibility of triple therapy with HCV protease inhibitors" or treatment according to the standard scheme of double interferon plus ribavirin combination therapy for patients with additional factors (except liver fibrosis) that reduce the likelihood of treatment with drugs of existing standard antiviral therapy.

DNA-Technology developed a kit (see Tables 67 and 68) for identification of interleukin 28B polymorphisms using real-time PCR technique.

Table 67. Interleukin-28B genetic polymorphisms

Gene	Gene function	Polymorphism	Identifier*	Possible genotypes	Association / Effects
IL 28 B – interleukin	It encodes cytokine that plays an important role in antiviral immune response. It is an interferon type III (IFNλ). It determines the probability of spontaneous HCV elimination and response to interferon plus ribavirin combination therapy	**C>T**	**rs12979860**	**C/C**	No abnormalities
				C/T	Reduced inducing activity in relation to Mx protein (they block primary transcription of viruses) 2-5-oligoadenylate synthase, ISGF3G.
				T/T	Reduced activation of the cytotoxic potential of CD8+ T cells. Reduced intensity of immune-mediated death of hepatocytes infected with HCV
		T>G	**rs8099917**	**T/T**	No abnormalities
				T/G	Reduced inducing activity in relation to Mx protein (they block primary transcription of viruses) 2-5-oligoadenylate synthase, ISGF3G.
				G/G	Reduced activation of the cytotoxic potential of CD8+ T cells. Reduced intensity of immune-mediated death of hepatocytes infected with HCV

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

Table 68. IL28B Real-Time PCR Genotyping Kit

Number of tests	48 tests
Kit format	Not pre-aliquoted
Taq-AT polymerase	2 tubes (24 mcl each)
Mineral oil	2 tubes (960 mcl each)
PCR buffer	2 tubes (480 mcl each)
Polymorphisms studied	IL28B: rs12979860 C>T – 960 mcl IL28B: rs8099917 T>G – 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.

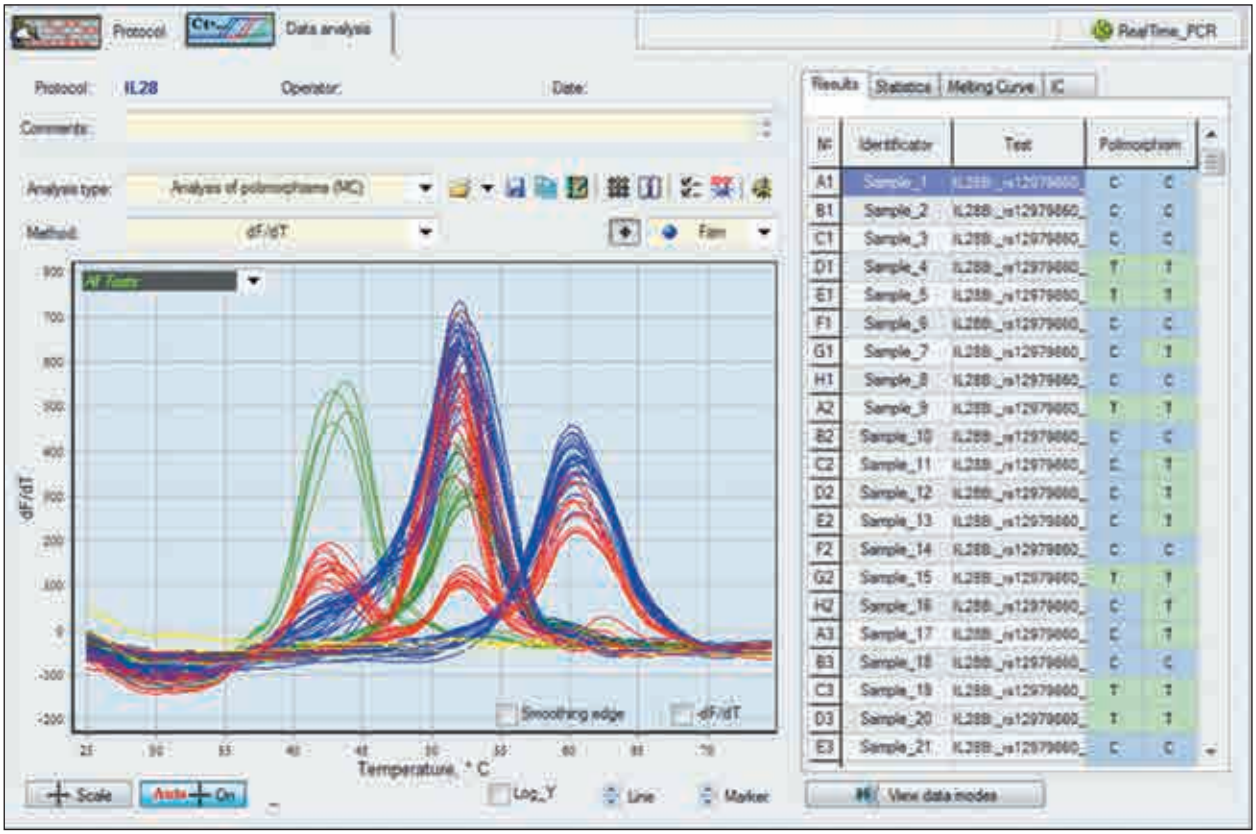
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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. 41).

A



B

№	Name of research	Results	
		Genotype	
1	IL28B:_rs12979860_C>T	T	T
2	IL28B:_rs8099917_T>G	T	G

Fig. 41. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

12.4.8. Osteoporosis Real-Time PCR Genotyping Kit

Osteoporosis is a progressive systemic skeletal disease characterized by decrease in the density and impaired micro-architectonics of bone tissue. It is accompanied by reduced bone strength and high risk of fractures. Osteoporosis is a leading cause of disability in the elderly, and is a contributing factor to fracture of the femoral neck, accompanied by high mortality. According to WHO, about 35 % of fractures in women and 20 % of fractures in men with osteoporosis are related to osteoporosis.

Identification of risk groups, early diagnosis of reduced bone mineral density (BMD) and osteoporosis prevention all help to reduce the risk of fractures and disability. However, in most countries, diagnosis of osteoporosis often comes after the fractures have occurred.

Among all osteoporosis cases, primary osteoporosis (mainly postmenopausal osteoporosis) accounts for about 85 %. Deficiency of sexual hormones observed in postmenopause, especially estrogens, is a significant risk factor. In addition to female gender and age, there are other risk factors for osteoporosis: Mongoloid or Caucasian race, low weight, tall height (over 172 cm for women and 183 for men), asthenic body build. Deficiency of 25-hydroxy vitamin D3 is an important risk factor for osteoporosis.

Adverse family history (presence of family relatives with osteoporosis) often indicates osteoporosis risk – indications of fragility fractures, especially hip and vertebrae fractures. High significance of genetic component in pathogenesis of the disease is shown: in 45-70 % of cases. Bone mineral density features are passed from parents to children [25, 118].

In some cases, despite the presence of normal bone mineral density when measured, there could be disorders in the microarchitectonics of bones that lead to increased fragility. This situation may lead to an underestimation of the risk of osteoporosis and dangerous fractures. In this regard, more and more attention is paid to detection of gene polymorphisms (SNP), associated with osteoporosis.

Gene network of osteoporosis

One of the best known of genes associated with osteoporosis is the **vitamin D receptor gene (VDR gene)**. Vitamin D receptor is an intranuclear hormone receptor for vitamin D3. This receptor is capable of altering the expression of genes involved in calcium homeostasis, which may lead to reduced bone mineral density in both children and young girls and in postmenopausal women. There is also a connection of low bone mineral density in men [45, 83, 102, 149].

Estrogens and estrogen receptors, among which the most important is the **estrogen receptor alpha (ESR1)**, play an important role in bone tissue metabolism. Estrogen receptors are presented both on osteoclasts and on osteoblasts. Estrogens inhibit bone resorption by reducing the amount, activity and lifetime of osteoclasts. Deficiency of estrogen or estrogen receptor gene polymorphism leads to disorder in remodeling with predominance of bone resorption over bone formation (Fig. 42).

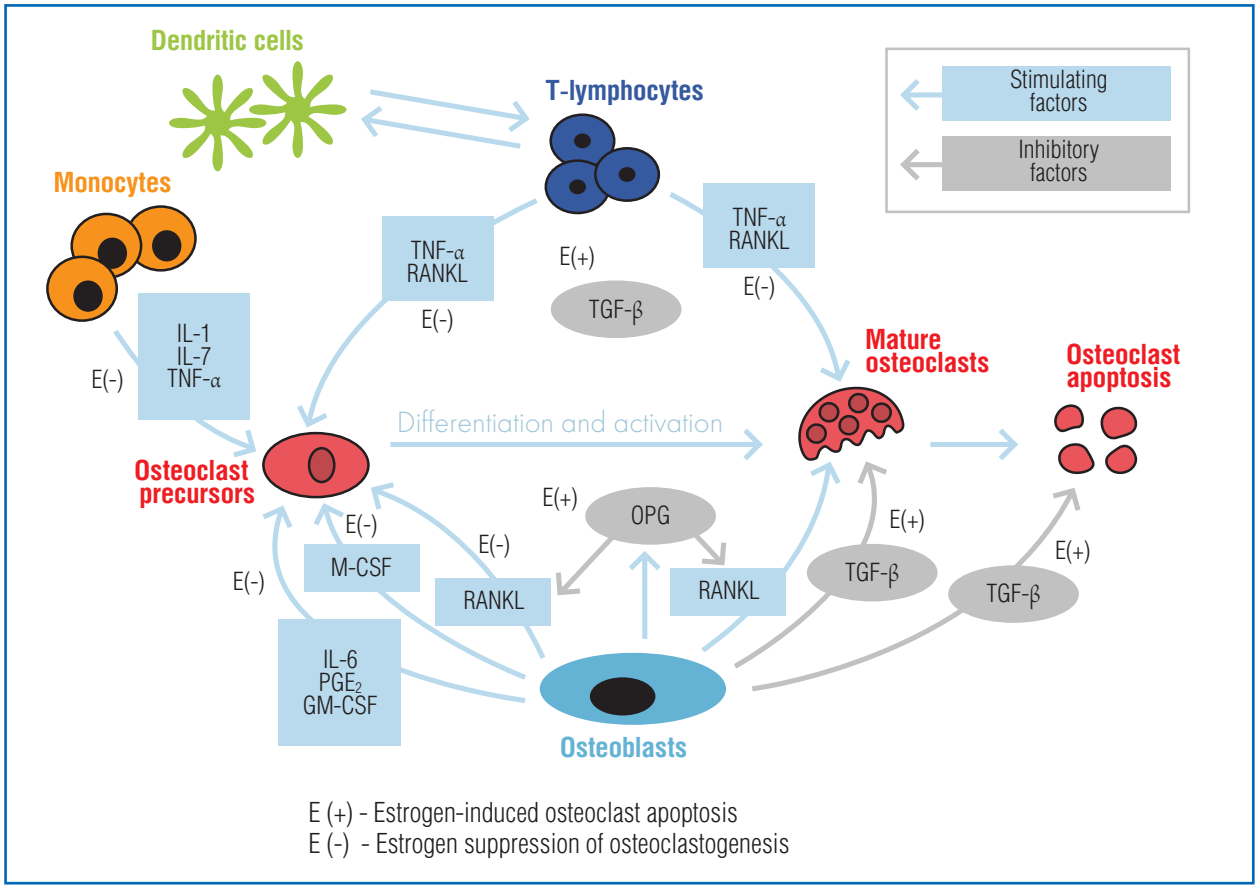


Fig. 42. Effect of estrogen and its deficiency on bone cells [111]

Aromatase enzyme (CYP19A1) plays an important role in synthesis of estrogens and in osteoporosis. It catalyzes conversion of testosterone to estrogens. It has been shown that aromatase levels affect the state of the spongy bone (spine), while the difference in the number of estrogen receptors affect the condition of predominantly cortical bone (femur neck) [94].

Imbalance of the **RANK/RANKL/OPG** system is a powerful factor leading to development of postmenopausal osteoporosis due to estrogen deficiency. The degree of bone remodeling is controlled by the balance between expression of osteoprotegerin (OPG) and expression of receptor activator of nuclear factor kappa-B ligand **(RANKL)**. When the balance shifts toward OPG, bone resorption is inhibited and bone formation dominates. In the case of prevailing influence of RANKL, and bone resorption prevails [76, 108, 141].

Production of proresorptive cytokines promotes osteoclastogenesis and inhibits osteoclast apoptosis. One of the most important cytokines (protein products of immune system cells), which influence bone metabolism is **interleukin-6 (IL-6)** amid existence of protective polymorphisms in which the risk of bone resorption and osteoporosis in postmenopausal women is reduced [43].

The bone tissue metabolism phase, which is independent of estrogens is activated with participation of **LRP5** (low-density lipoprotein receptor-related protein 5), which stimulates proliferation and differentiation of osteoblasts and osteoclasts. The defect of this gene is an independent risk factor for fracture, and does not depend on bone density, age and sex [108].

Disorder in collagen structure is one of the causes of fractures in postmenopausal osteoporosis that is also not associated with bone mineral density. Thank to collagen, the bone tissue combines hardness and durability with

elasticity and flexibility, while polymorphisms in the regulatory region of the COL1A1 gene (collagen, type I, alpha 1) encoding α-1 chain, leads to disorder in the collagen structure of bone and increase the risk of osteoporosis [50, 148].

It is important to bear in mind that the same parameter can have different effects in men and women. There is also a population dependency both in genetic and phenotypic manifestations (see Table 69) [107, 132, 137, 77]. For example, polymorphic locus -1997G/T of the COL1A1 gene is associated with BMD in most populations of the world. For example, according to a survey among Europeans, -1997 G allele carriers have higher BMD, while for women from the UK and Japan, the -1997 G allele is, on the contrary, associated with low BMD [82, 127, 146].

Table 69. Frequency of allelic variants of some genetic markers for osteoporosis in European and Asian populations [77]

Genes (loci)	Variants	Population	
		European	Asian
VDR 3'-end	BSM I	BB- 12-32 %, Bb -38-72 %, bb – 16-44 %	BB- 0-6.4%, Bb – 10-22%, bb -76.5-90 %
	Taq I	TT- 32-41 %, Tt-41-56 %, tt-6-22 %	TT- 75.2-90 %, Tt-10-23 %, tt- 0-1.5 %
	Apa I	AA-26-36 %, Aa - 43-54 %, aa 13.7-28 %	AA-3-13.6 %, AA – 28-50 %, aa -39-69 %
VDR start codon	For I	FF – 36 %, Ff –49 %, ff –15 %	FF – 33 %, Ff –53 %, ff –14 %
ESR	Pvu II (T397C)	PP – 18 %, Pp – 53 %, pp – 29 %	PP – 18 %, Pp – 52 %, pp – 30 %
	Xba I (C351G)	XX – 10 %, Xx – 48 %, xx – 42 %	XX – 5 %, Xx – 31 %, xx – 64 %
PTH (parathyroid hormone)	BSTB I	–	BB – 82 %, Bb – 17 %, bb – 1 %
Gglucocorticoid receptor (GR)	Asn363Ser	Asn Asn – 87.7-97.7 %, Asn Ser – 2.3-9.7 %, Ser Ser- 0-2.6 %	Asn Asn – 100 %
Calcitonin receptor (CTR)	C447-T	RR – 49 %, Rr – 44 %, rr – 7 %	–
	Taq I	TT – 11 %, Tt – 50 %, tt – 39 %	–
COLIA1	1a1-Sp1	SS – 48.8-70.7 %, Ss – 26.1-37.5 %, ss – 0-13.7 %	SS – 100 %
IL6	174 G/C	GG – 25.4-55.9 %, GC -35.1-52.8 %, CC – 7.9-25.4 %	GG – 74-100 %, GC – 0.7-24.3 %, CC – 0-2 %

Genetic studies in the Russian population also identified differences in the significance of certain polymorphisms, depending on regional and ethnic features.

According to Maltsev A.V., in a total sample of postmenopausal women from Volga-Ural region of Russia, markers of increased risk of osteoporotic fractures turned out to be the rs3102734 polymorphism genotype and the *T*T genotype of polymorphic variant rs3134069 of the OPG gene, genotype *C*T of polymorphic variant rs1801197 of the CTR gene [13].

At the same time, in women of Russian ancestry, markers of increased risk of fractures are *C*T genotype of polymorphic variant rs1801197 of the CTR gene and genotype *C*C of polymorphic variant rs9630182 of the PTH gene. While Tatar ethnic women with fracture risk are associated with *T*T genotype of the rs3102734 polymorphism of the OPG gene, *T*T genotype of the rs3134069 polymorphism of the OPG gene, haplotype *TG for loci rs3102735 and genotype Δ3/12 of polymorphism (TAAA)n repeats of the CYP19 gene, genotype *T*T of polymorphic variant rs3020314 of the ESR1 gene [13, 19].

Markers of reduced fracture risk are C*T* genotype of the rs3102734 polymorphism of the OPG gene and heterozygous genotype *T*G of polymorphic variant rs3134069 of gene OPG in the total sample. Moreover, among the Tatars,

markers of low-risk are also: *CGTG haplotype for loci rs3102734, rs2073617, rs2073618 and rs3134069, and *GG haplotype for loci rs3102734, rs2073617 of OPG gene, while for ethnic Russian women – C*T* genotype of the locus rs9630182 of the PTH gene [13].

Apart from genetic risk factors, non-genetic (environmental) factors play an important role in the formation of the disease [89]:

- *Non-modifiable factors:*
  - Low bone mineral density;
  - Female;
  - Over 65 years old;
  - Caucasian ethnicity;
  - Family history (osteoporosis or fragility fractures at the age of over 50 years);
  - Hypogonadism;
  - System intake of glucocorticoids for more than 3 months;
  - A history of fractures;
  - Prolonged immobilization
- *Modifiable factors*
  - BMI <18.5 kg/m^2, or body weight <57 kg;
  - Smoking;
  - Physical inactivity;
  - Inadequate intake of calcium;
  - Deficiency of vitamin D (also caused by lack of sun exposure);
  - Alcohol abuse

Existence of genetic risk factors is the basis for genetic analysis. Comprehensive assessment of genetic and non-genetic factors allows for evaluation of the risk and prognosis of the disease as accurately as possible and choose individual preventive and/or therapeutic measures.

Indications for genetic analysis:

- Osteoporosis, fragility and pathological fractures among close relatives
- Endocrine disorders that lead to menstrual disorders, diseases of the digestive system, circulatory system, and others
- Drugs (glucocorticoids)
- If there is non-genetic risk factors for osteoporosis: postmenopause, low weight, lack of exercise, frail body build, deficiency of 25-hydroxy vitamin D3, including alimentary, increased consumption of coffee, bad habits (alcohol, tobacco)
- Individual intolerance to lactose (milk products)

DNA-Technology developed a kit (see Tables 70 and 71) for identification of genetic polymorphisms associated with osteoporosis using real-time PCR method.

Table 70. Genetic polymorphisms associated with osteoporosis

Gene	Function of gene product	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
VDR – vitamin D3 receptor	The receptor is capable of altering the expression of genes involved in calcium homeostasis and immune response.	**283 A>G [BsmI]**	**rs 1544410**	**A/A**	No abnormalities
				A/G	Reduced bone mineral density, regardless of gender
				G/G	
		2A>G (Lys2Arg) [FokI]	**rs 2228570**	**A/A**	No abnormalities
				A/G	Reduced bone mineral density in children and postmenopausal women
				G/G	
ESR1 – estrogen receptor alpha	Ligand-activated transcription factor through which the action of estrogen is effected.	**-397T>C [PvuII]**	**rs2234693**	**T/T**	Depends on ethnicity
				T/C	
				C/C	
		-351 G>A [XbaI]	**rs 9340799**	**G/G**	No abnormalities
				G/A	Reduced bone mineral density starting from late puberty in girls. Reduced mineralization of the facial bones in postmenopausal women
				A/A	Reduced risk of overall bone fragility, especially vertebral fragility
RANKL – receptor activator of nuclear factor kappa-B ligand	Upon binding of RANK and RANK-ligand, mobilization of proliferation and activation of osteoclast occur.	**C>T**	**rs9594738**	**C/C**	No abnormalities
				C/T	Risk of disorders of bone remodeling
				T/T	
		C>T	**rs9594759**	**C/C**	No abnormalities
				C/T	Risk of disorders of bone remodeling
				T/T	
TNFRSF11B osteoprotegerin, steoclastogenesis inhibitory factor	Inhibits the binding of RANK and RANK-ligand, thereby inhibiting mobilization, proliferation and activation of osteoclasts	**245 A>C**	**rs3134069**	**A/A**	No abnormalities
				A/C	Increased bone fragility. Reduced bone density in the lumbar region in postmenopausal women
				C/C	
		163 (160) T>C	**rs 3102735**	**T/T**	No abnormalities
				T/C	Increased bone fragility. Reduced bone density in the lumbar region in postmenopausal women
				C/C	

Gene	Function of gene product	Polymorphism	Identifier*	Possible genotypes	Clinical manifestations
		4381 A>G	rs4355801	A/A	No abnormalities
				A/G	Reduced bone density and osteoporosis risk
				G/G	
LRP5 – low-density lipoprotein receptor-related protein 5	As part of Wnt-signaling pathway, stimulates proliferation and differentiation of osteoblasts and osteoclasts.	3989 C>T (Ala1330Val)	rs3736228	C/C	No abnormalities
				C/T	Osteoporosis and fractures
				T/T	
		1999 G>A (Val667Met)	rs4988321	G/G	No abnormalities
				G/A	Osteoporosis and fractures
				A/A	
COL1A1 – collagen, type I, alpha 1	The collagen is a protein base of connective tissues, including bone tissue.	-1997 C>A	rs1107946	C/C	No abnormalities
				C/A	Reduced bone mineral density. Association with osteoporosis
				A/A	
		1546 (6252) G>T [Sp1 S>s]	rs 1800012	G/G	No abnormalities
				G/T	Reduced bone mineral density. Association with osteoporosis
				T/T	
IL6 – interleukin-6 (IL-6)	IL-6 is a multifunctional cytokine involved particularly in bone metabolism.	-174 G>C	rs 1800795	G/G	No abnormalities
				G/C	Osteoarthritis risk
				C/C	Reduced risk of bone resorption and osteoporosis in postmenopausal women
CYP19A1 – aromatase	An enzyme that converts androgens to estrogens	C>T	rs936306	C/C	No abnormalities
				C/T	Osteoporosis risk
				T/T	
		A>G	rs2414096	A/A	No abnormalities
				A/G	Osteoporosis risk
				G/G	

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

Table 71. Osteoporosis 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	4 tubes (960 mcl each)
Polymorphisms studied	COL1A1: -1997 C>A – 1 tube (960 mcl) COL1A1: 1546 (6252) G>T [Sp1 S>s] – 1 tube (960 mcl) CYP19A1: A>G – 1 tube (960 mcl) CYP19A1: C>T – 1 tube (960 mcl) ESR1: -397 T>C [PvuII] – 1 tube (960 mcl) ESR1: -351 G>A [XbaI] – 1 tube (960 mcl) IL6: -174 G>C – 1 tube (960 mcl) LRP5: 1999 G>A (Val667Met) – 1 tube (960 mcl) LRP5: 3989 C>T (Ala1330Val) – 1 tube (960 mcl) RANKL: C>T [rs9594738] – 1 tube (960 mcl) RANKL: C>T [rs9594759] – 1 tube (960 mcl) TNFRSF11B (OPG): 245 A>C – 1 tube (960 mcl) TNFRSF11B (OPG): 4381 A>G [rs4355801] – 1 tube (960 mcl) TNFRSF11B (OPG): 163(160) T>C – 1 tube (960 mcl) VDR: 283 A>G [BsmI] – 1 tube (960 mcl) VDR: 2 A>G (Lys2Arg) [FokI] – 1 tube (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.

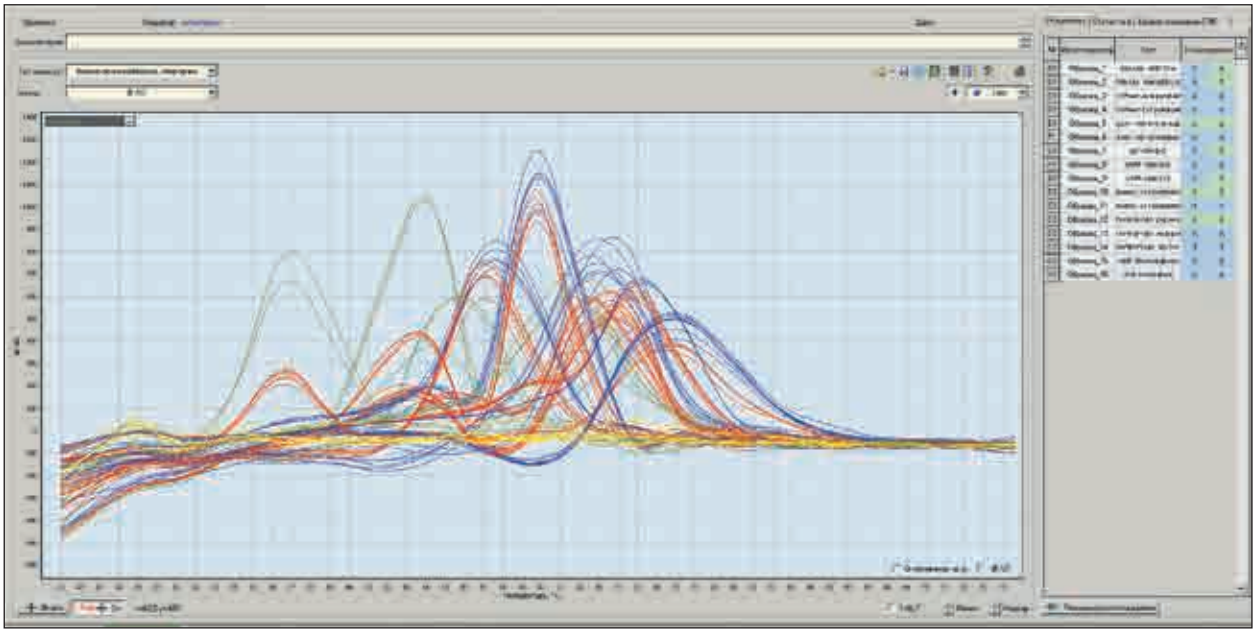
DNA extraction kits:
- *PREP-RAPID GENETICS;*
- *PREP-GS GENETICS*

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. 43).

A



B

№	Name of research	Results	
		Genotype	
1	COL1A1:_-1197_C>A	C	A
2	COL1A1:_1546 (6252)_G>T	G	T
3	CYP19A1:_A>G (rs2414096)	A	A
4	CYP19A1:_C>T (rs936306)	C	C
5	ESR1:_-397_T>C (Pvull)	C	C
6	ESR1:_-351_G>A (Xbal)	A	A
7	IL6:_-174_G>C	G	C
8	LRP5:_1999_G>A (Val667Met)	G	G
9	LRP5:_3989_C>T (Ala1330Val)	C	T
10	RANKL:_C>T (rs9594738)	T	T
11	RANKL:_C>T (rs9594759)	T	T
12	THFRSF11B (OPG):_245_A>C	C	C
13	THFRSF11B (OPG):_A>G (rs4355801)	A	A
14	THFRSF11B (OPG):_163 (160)_T>C	T	T
15	VDR:_283_A>G (Bsml)	G	G
16	VDR:_2_A>G (Fokl)	A	A

Fig. 43. Optical measurement analysis results
A – Optical measurement analysis (Fam channel)
B – Analysis report

VI. DNA/RNA PURIFICATION

VI. DNA/RNA PURIFICATION

13. KITS FOR NUCLEIC ACID EXTRACTION

The main objective of pre-analytical phase in molecular biological research is to ensure the safety and stability of biomaterial components and minimize fragmentation and loss of nucleic acids. One of the main ways of solving these problems is to correctly choose DNA and RNA extraction system.

DNA-Technology offers the following kits (see Table 72) for nucleic acid extraction:

- **Rapid DNA extraction:**
  - PREP-RAPID DNA Extraction Kit;
  - PREP-RAPID GENETICS DNA Extraction Kit;
- **Sorbent DNA extraction:**
  - PREP-GS DNA Extraction Kit;
  - PREP-GS-PLUS DNA Extraction Kit;
  - PREP-GS-Genetics DNA Extraction Kit;
- **Precipitation-based DNA/RNA extraction:**
  - PREP-NA DNA and RNA Extraction Kit;
  - PREP-NA-PLUS DNA and RNA Extraction Kit;
- **PREP-NA-FET DNA Extraction Kit**

Table 72. Kits produced by DNA-Technology for nucleic acid extraction

Name kit	Nucleic acids		Registration*
	DNA	RNA	
PREP-RAPID	*	–	RU/IVD
PREP-RAPID GENETICS	*	–	RU/IVD
PREP-GS	*	–	RU/IVD
PREP-GS-PLUS	*	–	RU/IVD
PREP-GS- Genetics	*	–	RU/IVD
PREP-NA	*	*	RU/IVD
PREP-NA-PLUS	*	*	RU/IVD
PREP-NA-FET	*	–	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

13.1. Rapid DNA extraction

The advantages of rapid DNA extraction method are:

- DNA extraction from samples within 15 minutes;
- Total DNA yield during cell lysis;
- Absence of additional washing significantly reduces the risk of DNA fragmentation and loss;
- Neutralization of inhibiting impurities.

13.1.1. PREP-RAPID DNA Extraction Kit

PREP-RAPID kit is designed for DNA extraction from biological material. It is also a medium for taking and storing samples. **Biomaterial transportation and DNA extraction are performed in one tube.**

The kit permits to perform the following:

- DNA extraction by lysis of cells and virus particles;
- Taking and storing (24 hours – at 2-8 °C, 2 weeks – at -20 °C) for transportation of the material;
- Inactivation of pathogenic microorganisms;
- Neutralization of inhibitors;
- Reduction of the number of necessary labware (when working with epithelial cell scrapings, the isolation process takes place in the same tube, in which the material was taken).

Kit format:
Single tubes (100 tubes, 500 mcl each).

Storage temperature: +2 to +8 °C.

Shelf life: 12 months.

Specimen for screening:

- Epithelial cell scrapings from the posterior pharyngeal wall, from the urethra, cervical canal, posterior vaginal vault, etc ;
- Saliva;
- Urine;
- Prostate secretion;
- Cerebrospinal fluid.

13.1.2. PREP-RAPID GENETICS DNA Extraction Kit

PREP-RAPID GENETICS DNA Extraction Kit is designed for DNA extraction from whole peripheral blood and subsequent genetic research by PCR. It provides collecting a large volume of human DNA: not less than 10 ng from 100 mcl of whole blood.

The kit (see Table 73) is optimized for DNA extraction from 48 analyzed samples (including negative control samples).

Table 73. PREP-RAPID GENETICS DNA Extraction Kit content

Reagent	Amount
Lysis buffer	1 vial – 28,8 ml
«PREP-RAPID» reagent	1 vial – 28,8 ml

Storage temperature: +2 to +8 °C.

Specimen for screening: Whole peripheral blood.

Shelf life: 6 months.

13.2. Sorbent DNA extraction

It is a universal method for isolating nucleic acids by cell lysing followed by DNA sorption on a carrier. It is effective when handling a wide spectrum of biomaterial. It secures minimum loss of DNA in the washing process through optimized composition of components of the lysing and washout solutions. It permits to neutralize inhibitors as much as possible and **can be recommended for the material containing significant amounts of impurities**.

13.2.1. PREP-GS DNA Extraction Kit

The kit permits to perform the following:

- Remove inhibiting impurities;
- Inactivate pathogens.

The kit (see Table 74) is optimized for **DNA extraction from 100 analyzed samples** (including negative control samples).

Table 74. PREP-GS DNA Extraction Kit contents

Reagent	Amount
Lysis buffer	1 vial – 15 ml
Sorbent	2 tubes – 1.0 ml
Washout solution № 1	1 vial – 20 ml
Washout solution №. 2	1 vial – 20 ml
Washout solution №. 3	1 vial – 20 ml
Elution solution	1 vial – 10 ml

Storage temperature: +2 to +8 °C.

Shelf life: 6 months.

Specimen for screening:

- Epithelial cell scrapings from the posterior pharyngeal wall, from the urethra, cervical canal, posterior vaginal vault, etc ;
- Blood plasma;
- Sperm;
- Prostate secretion;
- Cerebrospinal fluid;
- Urine;
- Minced tissue;
- Phlegm;
- Biopsy specimens;
- Saliva;
- Whey;
- *Material which contains significant amount of inhibiting impurities*

13.2.2. PREP-GS-PLUS DNA Extraction Kit

The kit is optimized for **DNA extraction from 50 analyzed samples** (including negative control samples). It is designed to receive a large volume of DNA (300 mcl) as compared with that when using the *PREP-GS* kit (50 mcl).

PREP-GS-PLUS (see Table 75) has increased the number of elution buffer compared with the PREP-GS kit.

The kit is recommended for DNA extraction and subsequent quantitative PCR analysis (biocenosis analysis using FEMOFLOR® kit, typing and quantification of human papillomavirus using *HPV QUANT, HPV 6/11 multiplex, HPV 16/18 multiplex* kits).

Table 75. PREP-GS-PLUS DNA Extraction Kit contents

Reagent	Amount
Lysis buffer	1 vial – 7.5 ml
Sorbent	2 tubes – 1.0 ml
Washout solution № 1	1 vial – 10 ml
Washout solution № 2	1 vial – 10 ml
Washout solution № 3	1 vial – 10 ml
Elution solution	1 vial – 5.0/15 ml

Storage temperature: +2 to +8 °C.

Shelf life: 6 months.

Specimen for screening:

- Epithelial cell scrapings from the posterior pharyngeal wall, from the urethra, cervical canal, posterior vaginal vault, etc ;
- Blood plasma;
- Sperm;
- Prostate secretion;
- Cerebrospinal fluid;
- Urine;
- Minced tissue;
- Phlegm;
- Biopsy specimens;
- Saliva;
- Whey;
- *Material which has significant amount of inhibiting impurities*

13.2.3. PREP-GS-GENETICS DNA Extraction Kit

PREP-GS-GENETICS is designed for DNA extraction from whole peripheral blood and subsequent genetic study by PCR. It provides collecting a large volume of human DNA: not less than 10 ng from 100 mcl of whole blood.

The kit (see Table 76) is optimized for **DNA extraction from 48 analyzed samples** (including negative control samples).

Table 76. PREP-GS-GENETICS DNA Extraction Kit content

Reagent	Amount
Lysis buffer	1 vial – 7.2 ml
Sorbent	1 tube – 960 mcl
Washout solution № 1	1 vial – 19.2 ml
Washout solution № 2	1 vial – 9.6 ml
Washout solution № 3	1 vial – 9.6 ml
Elution solution	1 vial – 14.4 ml

Storage temperature: +2 to +8 °C.

Shelf life: 6 months.

Specimen for screening: Whole peripheral blood.

13.3. Precipitation-based DNA/RNA extraction

Universal **method for obtaining DNA and RNA** based on lysis and subsequent precipitation of nucleic acids from a wide spectrum of biomaterial for subsequent analysis by reverse transcriptase (RNA) and/or polymerase chain reaction (DNA).

Simultaneous extraction of maximum amount of DNA and RNA from a sample is advantage when handling samples containing a small amount of the material. This nucleic acid isolation method provides optimal conditions for quantitative PCR analysis, primarily in determining viral load.

13.3.1. PREP-NA DNA and RNA Extraction Kit

The kit (see Table 77) is designed for **DNA/RNA extraction from 100 analyzed samples** (including negative control samples). It is possible to concentrate RNA by reducing the volume of elution buffer, which increases efficiency and diagnostic sensitivity of PCR analysis.

It is recommended for the study of clinical material for the presence of infections caused by RNA- and DNA-containing viruses.

Table 77. PREP-NA DNA and RNA Extraction Kit content

Reagent	Amount
Lysis buffer	1 vial – 30 ml
Precipitation buffer	1 vial – 40 ml
Washout solution № 1	1 vial – 50 ml
Washout solution № 2	1 vial – 30 ml
Dilution	4 tubes – 1.25 ml
Negative control sample («C-»)	2 tubes – 1.5 ml
Internal control sample (RNA-IC)	1 tube – 1 ml
Internal control sample (DNA- IC)	1 tube – 1 ml

Storage temperature: +2 to +8 °C.

Shelf life: 12 months.

Specimen for screening:

- Epithelial cell scrapings from the posterior pharyngeal wall, from the urethra, cervical canal, posterior vaginal vault;
- Blood plasma;
- Phlegm;
- Prostate secretion;
- Saliva;
- Urine;
- Cerebrospinal fluid;
- Sperm;
- Milk;
- Smears and swabs from the nasal cavity and oropharynx;
- Faeces;
- Material from sick and dead animals (smears and swabs from the trachea, nasal cavity, pharynx, cloaca, faeces; internal organs).

13.3.2. PREP-NA PLUS DNA and RNA Extraction Kit

The kit is optimized for **DNA/RNA extraction from 50 analyzed samples** (including negative control samples). It is designed for collecting large volume of DNA (300 mcl) as compared with that when using kit PREP-NA.

PREP-NA PLUS DNA and RNA Extraction Kit has increased number of elution buffer compared with the PREP-NA kit (see Table 78).

The kit is recommended for DNA extraction and subsequent quantitative PCR analysis (biocoenosis analysis using *FEMOFLOR®* kit, typing and quantification of human papillomavirus using *HPV QUANT* kit).

Table 78. PREP-NA PLUS DNA and RNA Extraction Kit content

Reagent	Amount
Lysis buffer	1 vial – 15 ml
Precipitation reagent	1 vial – 20 ml
Washout solution № 1	1 vial – 25 ml
Washout solution № 2	1 vial – 15 ml
Dilution	1 vial – 15 ml

Storage temperature: +2 to +8 °C.

Shelf life: 12 months.

Specimen for screening:

- Epithelial cell scrapings from the posterior pharyngeal wall, from the urethra, cervical canal, posterior vaginal vault, etc;
- Peripheral blood;
- Phlegm;
- Prostate secretion;
- Saliva;
- Urine;
- Cerebrospinal fluid;
- Sperm;
- Milk.

13.4. PREP-NA-FET DNA Extraction Kit

The unique PREP-NA-FET DNA Extraction Kit is intended for extraction of fetal DNA (DNA of fetus) from the mother's blood plasma.

The high quality of extraction is achieved due to:

- The ability of using a big volume of plasma;
- Removing from the sample the major amount of protein on the first step;
- Obtaining the maximum highest concentration fraction of fetal DNA.

The kit is designed for the extraction of **fetal from the 50 analyzed samples** (including negative controls) (Table 79).

Table 79. The content of PREP-NA-FET DNA Extraction Kit

Reagent	Amount
Lysis buffer	1 vial - 15 ml
Precipitation buffer	1 vial (30 mL)
Washout solution № 1	1 vial (25 mL)
Washout solution № 2	1 vial (15 mL)
Dilution buffer	2 tubes (1.4 mL in each tube)
Negative control ("C–")	1 vial (13.5 mL)
Deproteinization solution	1 tube (1.0 mL)

Storage temperature: +2 to +8 °C.

Shelf life: 12 months

Specimen for screening:

- Peripheral blood

ATTENTION!

The additional equipment is necessary:

- centrifuge for 4.5 mL tubes with maximal speed not lower than 1150 g;
- cooling tube rack or container.

14. SIC. SAMPLE INTAKE CONTROL REAL-TIME PCR KIT

The bulk of PCR diagnostic error occurs in the pre-analytical phase and depends on how the following is carried out:

- *Correct location of region for taking biological material for study* (the place of proposed localization of infection).
- *Correct collection of biological material*.

Even with correct location of biomaterial collection region, it is necessary to consider the fact that the region should contain the highest concentration of the microorganisms in question and not have undesirable PCR-inhibiting impurities.

- *Processing of biological material*.
- *Storage of biological material*.

Besides, the probability of errors is high in the analytical phase, primarily due to *wrong choice of sample preparation system* – choice of isolation method should be determined by the nature of the biological material and by the degree of its pollution by potential PCR inhibitors. Defective nucleic acid extraction technology leads to substantial losses and makes it impossible to obtain the correct result.

All of these factors contribute to the probability of obtaining false negative results, reduce the quality of laboratory work, increase the use of reagents needed for re-analysis.

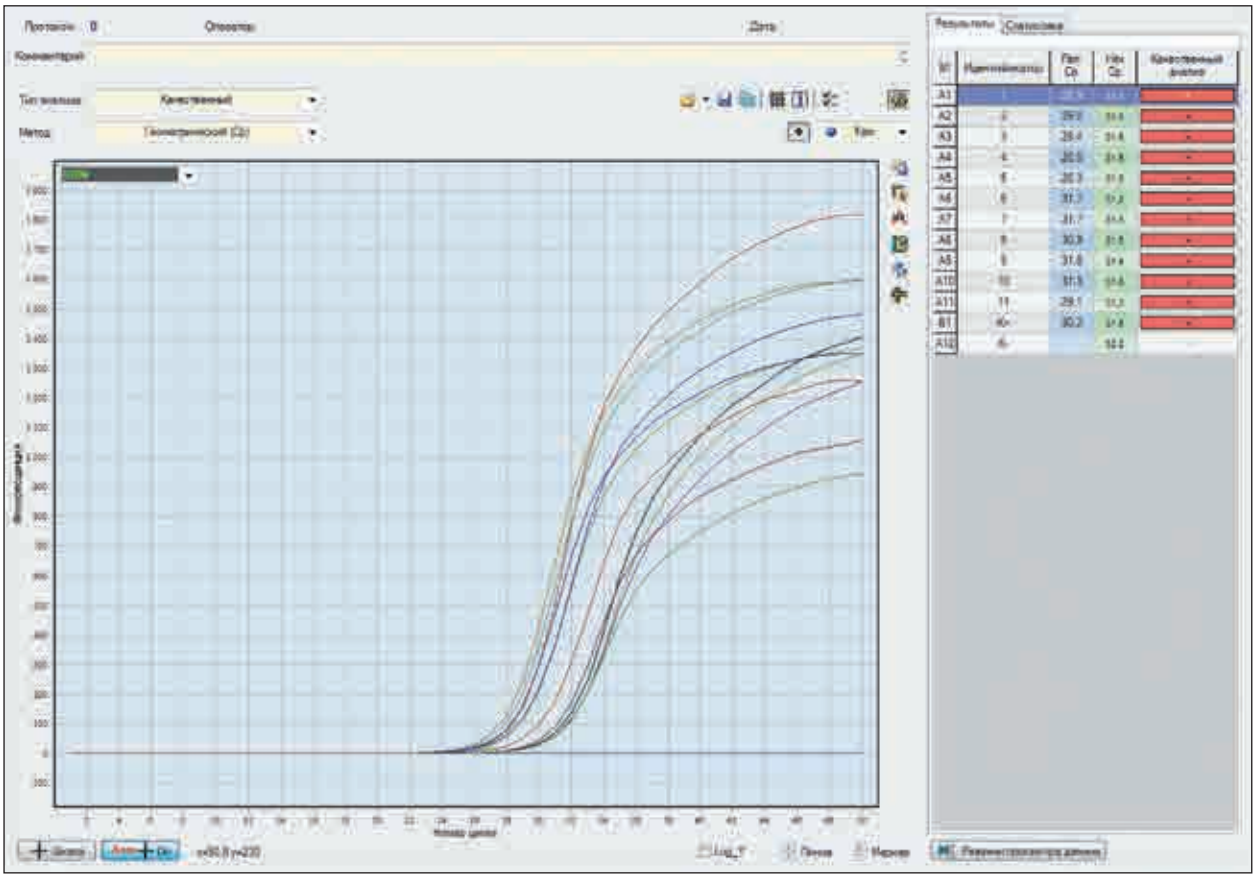
Usage of SIC kit permits to estimate the approximate amount of isolated DNA directly through PCR results (Fig. 44, Table 80).

SIC kit is recommended to be used:

- To avoid false-negative results in the study of a biological material that contains human epithelial cells (to control collection of material by the clinician);
- For controlling the amount of human genomic DNA extracted from any kind of material.

The kit can be used in clinical diagnostic laboratories of medical institutions and in research practice.

A



B

Number of the hole	Identificator of the tube	Cp, Fam	Cp, Hex	Result
A1	1	28,9	31,2	+
A2	2	29,0	31,4	+
A3	3	28,4	31,6	+
A4	4	28,0	31,6	+
A5	5	28,3	31,3	+
A6	6	31,7	31,2	+
A7	7	31,7	31,3	+
A8	8	30,9	31,5	+
A9	9	31,8	31,4	+
A10	10	31,3	31,6	+
A11	11	29,1	31,3	+
B1	K+	30,2	31,5	+
A12	K–		32,2	–

Fig. 44. Optical measurement analysis results

A – Optical measurement analysis (Fam channel)

B – Analysis report

Table 80. Interpretation of PCR results

Cp on the channel Fam	Cp on the channel Hex	Interpretation	Amount of genomic DNA per reaction, ng
< 23	not considered	Human DNA presents in **sufficient** amount	> 750
23-32	not considered		750-1,0
32-38	not considered	Human DNA presents in sufficient amount **(excluding genotyping)**	1,0-0,01
> 38	27-32	Human DNA presents in **insufficient** amount	< 0,01
> 38	> 32	Possibly, DNA preparation contains PCR inhibitors	< 0,01
not defined	27-32	Human DNA is absent or presents in trace amounts	–
not defined	not considered or 32	Unreliable result	–

DNA-Technology offers the following kit (see Table 81) for PCR amplification of human genomic DNA using real-time PCR technique.

Table 81. Sample Intake Control Real-Time PCR Kit

Name	Detection format				Registration*
	Forez	Flash	Rt	qPCR	
SIC	–	–	*	–	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);
- Single tubes (0.2 ml).

Storage temperature: +2 to +8 °C.

Shelf life: Rt – 12 months.

Recommended DNA extraction kits:
- PREP-RAPID
- PREP-RAPID GENETICS
- PREP-GS
- PREP-GS-PLUS
- PREP-GS- Genetics
- PREP-NA
- PREP-NA-PLUS

Specimen for screening:
- Urethral scrapings;
- Conjunctival scrapings;
- Scrapings from the back wall of the nasopharynx and other mucous membranes;
- Urine cell sediment;
- Blood and others.

Equipment required for analysis:
- DT devices produced by DNA-Technology (DTlite, DTprime, DT-96);
- IQ Cycler devices produced by Bio-Rad Laboratories and Rotor-Gene devices produced by QIAGEN

Software:

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

Notes:

Address:
Varshavskoe shosse (high-way), 125Zh, Bld. 6, fl. 5, Moscow, 117587, Russia
Phone: +7 (495) 640-17-71

Web: www.dna-technology.ru
General sales: sales@dna-technology.ru