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INSTRUCTION

Kit for mutations detection in *BRAF* gene based on real-time PCR in the probe of human genomic DNA from paraffin-fixed tissue samples (Test-BRAF-tissue)

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Introduction

Target analyte is *BRAF* gene considered when examining patients with Stage III-IV metastatic melanoma for qualitative detection of V600E, V600E complex mutations status (only detect V600E or V600E complex, not differ) and V600K for determination of indications for targeted therapy with inhibitors of the kinase activity of BRAF and MEK.

Scientific validation.

Melanoma remains the most deadly form of malignant skin disease with high risk of metastases. Metastatic melanoma is prognostic highly unfavorable and resistant to traditional chemotherapy and biologic treatment. There is a great progress in understanding of the molecular mechanisms underlying melanoma initiation and progression.

In 2002 scientists identified the high frequency of BRAF cancer-causing mutations from 62% to 72%. But at early disease BRAF mutations detect only disease progression that have important prognostic value.

The *BRAF* gene (7q34) encodes serine-threonine kinase whose mutations in the activating domain cause stable cascade hyperactivation of mitogen-activated protein kinases MEK and ERK.

The main cell signaling pathways and oncogene driver mutations are involved in melanoma pathogenesis. RAS/RAF/MEK/ERK cascade is hyperactivated in 75 % of cutaneous melanoma cases.

Activating *BRAF* mutations are found in 50-60% of skin melanoma cases, whereas in melanoma with chronic insolation – 11%, in acryl melanoma – 23%, melanoma of mucous membranes – 11%.

Timely diagnosis in the early stages of the disease and removal of the tumor nidus are the main factors that allow to make melanoma treatment as successful as possible.

When mutation V600E of *BRAF* gene was discovered, it was made good progress in studying the molecular mechanisms of melanoma carcinogenesis. This led to the development of new molecular-targeted drugs.

Since 2002, when the BRAF V600E mutation was discovered, significant progress has been made in studying the molecular mechanisms of melanoma carcinogenesis. This led to the creation of new medicinal

drugs, molecularly-targeted drugs such as dabrafenib, vemurafenib, trametinib.

Dabrafenib and vemurafenib are BRAF kinase activity inhibitors effective in treating patients with metastatic melanoma with BRAF V600E or V600K mutations. The results of studies of BRAF inhibitors, which showed their high efficacy at metastatic melanoma and a favorable safety profile made it possible to prefer these drugs instead chemotherapy and in cerebral metastases. Today there are published the research data that confirm the effectiveness of vemurafenib in cerebral melanoma metastases with an activating mutation BRAF V600.

Target drugs are prescribed as monotherapy or in complex depend on special clinical situation.

Tramenitib and selumetinib MEK inhibitors improve patients survival with V600E/K mutations with BRAF inhibitors. Dual inhibition of the MAPK signal pathway while simultaneous use of BRAF and MEK inhibitors is more effective in comparison with BRAF inhibitor only. It allows to prolong survival and improve acceptability of the treatment that leads to reduction of secondary carcinoma.

Use of BRAF/MEK inhibitors for patients with metastatic melanoma having BRAF mutation is the first successful example of personalized therapy. These drugs replaced chemotherapy and became the new standard for melanoma treatment with BRAF mutation. It confirms that it is necessary to develop different treatment approaches for various molecular genetic subtypes of melanoma.

Application area - clinical laboratory diagnostics, oncology.

Indications and contra indications for usage.

Indications for usage: The kit «Test-BRAF-tissue» is recommended when examining patients with Stage III-IV metastatic melanoma for qualitative detection of mutations V600E, V600E complex (only detect V600E or V600E complex, not differ) and V600K for determination of indications for targeted therapy.

Contra indications for usage: none.

1. Intended use

Intended use: the kit «Test-BRAF-tissue» is intended for qualitative detection of V600E, V600E complex mutations status (only detect V600E or V600E complex, not differ) and V600K of the *BRAF* gene by real-time allele-specific PCR in human genomic DNA from paraffin-fixed tissue samples for indication of targeted therapy. The therapy is conducted using BRAF and MEK inhibitors of the kinase activity of when examining patients with Stage III-IV metastatic melanoma with BRAF mutations.

Functional use – obtained results may be used when examining patients with Stage III-IV metastatic melanoma.

Potential users of the medical device.

The kit of reagents is intended for professional use in the medical centers and clinical diagnostic laboratories with oncological specialization. Professional level of potential users is doctor of the clinical laboratory medicine, medical laboratory technician.

2. Method principle

Method

Allele-specific real-time PCR.

Type of test sample.

Material for PCR reaction is human genomic DNA from samples of paraffin-fixed tissue.

Determination principle

Qualitative detection of mutations V600E, V600E complex (detect V600E, V600E complex mutations, not differ) and V600K of the *BRAF* gene by allele-specific real-time PCR in human genomic DNA from paraffin-fixed tissue samples includes three stages:

1) PCR preparation;
2) DNA PCR-amplification and real-time hybridization-fluorescent detection;
3) Interpretation of results.

Genomic DNA samples from paraffin-fixed tissue are used for amplification reactions of *BRAF* gene sites in the reaction buffer, using

primers specific for these DNA sites and Taq-polymerase enzyme. The reaction mixture for amplification includes allele-specific fluorescent-labeled oligonucleotide probes that are hybridized with the complementary site of the amplified DNA-target and destroyed by Taq-polymerase resulting increased fluorescence intensity. This enables to observe specific amplification product accumulation by measuring the fluorescent signal intensity. The fluorescent signal is detected directly during PCR using a cycler with a system for fluorescent signal detection in «real-time» mode.

PCR products of BRAF gene are identified in 5'-exonuclease reaction with FAM and HEX label probes.

DNA amplification product of normal variant of *BRAF* gene is detected by the channel conforming **HEX** fluorophore. DNA amplification product of mutant variant of *BRAF* gene is detected by the channel conforming **FAM** fluorophore.

Method limitations

The kit «Test-BRAF-tissue» is intended for detection of V600E, V600E complex and V600K mutations status in patients with Stage III-IV metastatic melanoma.

The kit of reagents «Test-BRAF-tissue» detects V600E and V600E complex mutations status but does not differ them. Obtained results are used for determination of indications to the targeted therapy by BRAF and MEK inhibitors of kinase activity according to clinical guideline «Skin and mucosal melanoma» dd. 2018 (approved by Ministry of Health of the Russian Federation).

Mutations detection depends on sample integrity and amplified DNA amount. Purity of extracted DNA expressed in ratio A260/280 nm must be not less than 1,4. DNA concentration enough for testing must be 1-100 ng/μl.

Tumor tissue is not homogeneous, testing results obtained from tissue sample may not coincide with results obtained from other tissue areas. And tumor samples may have a normal tissue (non-tumor). When using genomic DNA samples isolated from tissue without tumor, the kit «Test-BRAF-tissue» is not able to detect mutations of *BRAF* gene.

PCR is sensitive to contamination. Use caution to avoid contamination of DNA samples and reaction mixes by tubes with PC (positive control sample) or PCR products.

The kit of reagents «Test-BRAF-tissue» may not be used for diagnostics of any pathology. It is intended for qualitative detection of V600E, V600E complex mutations status (only detect V600E or V600E complex, not differ) and V600K of the *BRAF* gene by real-time allele-specific PCR in human genomic DNA from paraffin-fixed tissue samples for indication of targeted therapy. The therapy is conducted using BRAF and MEK inhibitors of the kinase activity of when examining patients with Stage III-IV metastatic melanoma with BRAF mutations.

Total time of the analysis procedure is 2-2.5 hours.

3. Kit contents

The kit is produced in one version – «Test-BRAF-tissue». Each kit «Test-BRAF-tissue» contains reagents for 24 tests.

Kit contents

Kit «Test-BRAF-tissue» includes:

Table 1 – Contents of the kit «Test-BRAF-tissue»

No.	Reagent	Marking on the tube cap	Description	Tube quantity, volume, µl
1	PCR Mix V600E/Ec	**V600E/Ec**	Pink-colored transparent liquid	1 tube (120 µl)
2	PCR Mix V600K	**V600K**	Pink-colored transparent liquid	1 tube (120 µl)
3	PC	**К+**	Transparent colorless liquid	1 tube (120 µl)
4	NTC	**К-**	Transparent colorless liquid	1 tube (120 µl)
5	Taq-polymerase	**Taq**	Transparent colorless liquid	1 tube (580 µl)

Positive control sample (PC) is ready-to-use and is the mix of genomic DNA from Jurkat human cell culture with concentration of 400 GE/ µl (genome-equivalent per µl) of *BRAF* gene and artificial-synthesized insertion with 300 bps size in plasmid vector pAL-TA with concentration 20 GE/µl containing equimolar mixture of mutations

V600E and V600E complex. It contains 5% of mutant and 95% of normal DNA copies.

Deionized water is used as NTC.

All reaction mixes contain HEX-labeled internal control (see Section 11 «Recording and interpretation of the results»). It is control of DNA isolation effectiveness and possible inhibition in the probe that may cause false-negative results.

The kit does not include any medicines, materials of human or animal origin.

4. Kit characteristics

4.1 Technical and functional characteristics

Table 2 – Technical and functional characteristics of the kit «Test-BRAF-tissue»

Name	Characteristics and requirements
1. Technical characteristics	
1.1 Appearance	
PCR mix V600E/Ec, **V600E/Ec**	Pink-colored transparent liquid
PCR mix V600K, **V600K**	Pink-colored transparent liquid
PC, **К+**	Transparent colorless liquid
NTC, **К-**	Transparent colorless liquid
Taq-polymerase, **Taq**	Transparent colorless liquid
1.2. Configuration	According to p. 1.4 in TS 21.20.23-007-97638376-2017
1.3. Marking	According to p. 1.5 in TS 21.20.23-007-97638376-2017
1.4. Packing	According to p. 1.6 in TS 21.20.23-007-97638376-2017
2. Functional characteristics	
2.1. Positive results with PC	Registration of the fluorescence signal rise with Ct≤ 35 in tubes with PC on the channels FAM and HEX

Name	Characteristics and requirements
2.2. Negative results with NTC	No fluorescence signal rise in tubes with NTC on the channels FAM and HEX

4.2 Analytical characteristics

Table 3 – Analytical characteristics of the kit «Test-BRAF-tissue»

Analytical specificity	Specific to mutations V600E, V600E complex (detect V600E/V600E complex, but not differ) and V600K of the *BRAF* gene
Analytical sensitivity	10 copies of *BRAF* gene to 1 µl of DNA solution

Specificity of the testing is determined by oligonucleotide primers matched to homologous genes regions and by specific fluorescent oligonucleotide probes for hybridization with complementary regions of amplicons (specific amplification products). It excludes cross-reactions.

List of detected mutations ID mutations is shown in the Table 4 below (the kit detects V600E/V600E complex, but does not differ).

Table 4 – List of mutations and COSMIC identities*

Mutation	Base change	Amino acids change	COSMIC ID*
V600E complex	c.1799_1800TG>AA (Complex)	p.V600E	475
V600E	c.1799T>A	p.V600E	476
V600K	c.1798_1799GT>AA	p.V600K	473

* COSMIC IDs are taken from the Catalog of Somatic Mutations in Cancer: (www.sanger.ac.uk/genetics/CGP/cosmic).

Precision was studied under conditions of repeatability and reproducibility to ensure analytical accuracy. The indicator values confirmed the high repeatability and reproducibility of the results.

5. List of risks

Border risk zone includes:

- loss of functional properties of the reagents included in the kit, due to transportation, storage or operation under inappropriate conditions,
- utilization of the kit with violation of safety and deactivation measures;
- crossover contamination of the samples;
- contamination of materials by inhibitors;
- contamination of reaction mixes with DNA samples by PC tube or PCR products;
- failure to meet the requirements for sample preparation, testing procedure and utilization because of unqualified personnel;
- use of invalid kits (use after shelf life expiration or packaging violation).

No risks have been identified in the unacceptable zone.

The total residual risk of using the medical product «Kit for mutations detection in *BRAF* gene based on real-time PCR in the probe of human genomic DNA from paraffin-fixed tissue samples (Test-BRAF-tissue) by TS 21.20.23-007-97638376-2017» produced by TestGene LLC, is acceptable, and the benefit of its using exceeds the risk.

6. Precautions for handling

Class depending on the potential risk of use is 2b in accordance with the nomenclature classification of medical devices approved by order of the Ministry of Health of the Russian Federation dd. 06.06.2012 N 4n.

All components and reagents contained in composition of «Test-BRAF-tissue» are of 4 hazard class (low-hazard substances) in accordance with GOST 12.1.007-76 «Occupational safety standards system. Noxious substances. Classification and general safety requirements».

Reagents included in «Test-BRAF-tissue» kit have low vapor pressure and eliminate the possibility of inhalation toxicity.

Reagents included in «Test-BRAF-tissue» kit are not toxic as they are prepared by mixing of separate nontoxic components.

The work shall be carried out in a laboratory performing bimolecular (PCR) testing of clinical material in compliance with the sanitary and epidemiologic rules SanPiN 2.1.7.2790-10 «Sanitary and Epidemiologic Requirements to the address with Medical Waste Products». The following requirements should always be fulfilled during the work:

- Dispose of unused reagents in accordance with p. 4.28 of SanPiN 2.1.7.2790-10 «Sanitary and Epidemiologic Requirements to Handling of Medical Waste».

ATTENTION! When discarding waste products after amplification (tubes containing PCR products), it is not allowed to open the tubes and spray their contents because this may result in contamination of the laboratory area, equipment and reagents with PCR products.

- Use the kit strictly for its intended use as per this instruction.
- Admit only specially trained staff to the work with the kit.
- Do not use the kit after the expiry date.
- Avoid contact with skin, eyes and mucous membranes. In case of contact, wash immediately the affected area with water and seek medical attention.

The necessary precautions regarding the influence of magnetic fields, external electric actions, electrostatic discharges, pressure or pressure drops, overload, sources of explosion or ignition are not provided.

As a part of the kit, there are no substances of human or animal origin that have a potential infectious nature. Therefore precautions against any special, unusual risks are not provided during using or implementation of the product.

7. Required equipment and materials

Equipment:

1. PCR-box (e.g., BAV-PCR-Laminar-S, Laminar Systems, Russia).
2. Vortex (e.g., TETA-2, Biocom, Russia).
3. Kit of electronic or automatic variable volume dispensers (e.g., Eppendorf, Germany).
4. Refrigerator with a temperature from 2 °C to 8 °C with a freezing chamber (max minus 16 °C).
5. Cycler of rotary type, e.g., Rotor-Gene 3000 or 6000 (Corbett Research, Australia), or cycler of plate type, e.g. Real-Time CFX96 Touch (e.g., BioRad, USA), DT-Prime (DNA-Technology, Russia) or equivalent ones.

Materials and reagents not included in the kit:

ATTENTION! When working with DNA it is necessary to use only sterile disposable plastic consumables with a special marking «DNase-free».

1. Disposable tips with an aerosol barrier of up to 200 µl, up to 100 µl, up to 20 and 10 µl (e.g., Axygen, USA).
2. Racks for tips (e.g., Axygen, USA) and 0.5 (0.2 ml) microtubes (e.g., InterLabService, Russia).
3. An individual coat and disposable gloves.
4. Container with a cover for disinfecting solution.
5. Disposable polypropylene tubes for PCR:

a) 0.2 ml tubes (flat cap, not stripped), (e.g., Axygen, USA) for placing in a rotor for 36 tubes – for devices for real-time PCR with detection through the tube bottom (e.g., Rotor-Gene).

b) 0.2 ml tubes (domed cap) (e.g., Axygen, USA) for devices for real-time PCR with detection through the cap (e.g., CFX96, DT-Prime).

6. PCR plates (may be used instead of the tubes identified in p.5).
7. Optically transparent film for sealing the plates.

8. Test samples

Material for PCR reaction is human genomic DNA from samples of paraffin-fixed tissue.

8.1 Obtaining of human genomic DNA from samples of paraffin-fixed tissue

For isolation of human genomic DNA from paraffin-fixed tissue which is necessary for PCR purity testing, it is recommended to use the following kits of reagents:

- Kit for human genomic DNA isolation from formalin-fixed paraffin-embedded (FFPE) tissue (DNA-Tissue-F) by TS 21.20.23-009-97638376-2016 in the versions: 1) «DNA-Tissue-F-50» for 50 tests, 2) «DNA-Tissue-F-100» for 100 tests (TestGene, Russia).
- NucleoSpin FFPE DNA (MACHEREY-NAGEL, Germany)
- QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany)

or equivalent for DNA isolation from tissue with the following characteristics:

- DNA purity expressed in ratio A260/280 nm must be not less than 1,4.
- DNA concentration enough for testing must be 1-100 ng/μl.

Applicability criteria of the histologic specimens for DNA isolation and subsequent testing of tumor cells

According to the results of morphological testing tumor should be not less than 60% of tissue in the cut section of FFPE block.

According to the results of morphological testing necrotic and hemorrhage zones should be not more than 15% of tissue in the cut section of FFPE block.

In case if the sample fails to meet requirements of criteria, it is recommended to use other sample.

Testing material not to be used in case of violation of storage and transportation conditions (temperature, duration, repeated freezing-thawing).

It is necessary to minimize risk of samples cross-contamination when preparing cutting sections from FFPE block.

Precautions measures:

- Use disposable no-powder gloves;
- Test in PCR-box or in laminar flow unit;
- Use disposable microtome blades and sterile forceps;
- Dispose first two cut sections, for testing use cut sections beginning with third;
- Do not place cut sections in a water bath.

8.2 Interfering substances and limitations of sampling material use

Impact of potentially interfering substances on performance of the kit «Test-BRAF-tissue» was checked using DNA isolated by Kit for human genomic DNA isolation from formalin-fixed paraffin-embedded (FFPE) tissue (DNA-Tissue-F) by TS 21.20.23-009-97638376-2016 in the versions: 1) «DNA-Tissue-F-50» for 50 tests, 2) «DNA-Tissue-F-100» for 100 tests (TestGene, Russia).

For evaluating the impact of potentially interfering substances it was studied by analyzing the effect of each substances on C_t values and identification of mutations status in the test sample, at two concentrations (maximum and minimum).

According to intended use the material for PCR is human genomic DNA from samples of paraffin-fixed tissue.

In this case interfering substances may come from the following sources:

1. Substances added during sample preparation (additives, stabilisers) – paraffin used for FFPE-blocks preparing.

2. Substances found in specific types of samples – tissue contamination by hemoglobin that can inhibit PCR if not sufficiently purified during isolation.

3. Kit components for DNA isolation from tissue (for human genomic DNA samples from paraffin-fixed tissue it is suggested to use the kit of reagents for isolation of human genomic DNA from FFPE tissues).

Potentially interfering substances and its concentrations listed in the table 4.

Table 4 - Potentially interfering substances and its concentrations

Interfering substances (Substances type)	Maximum concentration (µl/ 200 µl of DNA solution)	Minimum concentration (µl/ 200 µl of DNA solution)
Paraffin xylol	$2{,}00*10^{-4}$	$5{,}00*10^{-5}$
Xylol	$2{,}00*10^{-4}$	$5{,}00*10^{-5}$
Ethanol (95%)	$1{,}35*10^{-3}$	$3{,}38*10^{-4}$
DNA Binding buffer	$5{,}40*10^{-4}$	$1{,}35*10^{-4}$
Proteinase K	$1{,}32*10^{-5}$	$3{,}30*10^{-6}$
Eluent	$1{,}33*10^{-3}$	$3{,}33*10^{-5}$
Wash buffer №1	0,50	$1{,}25*10^{-1}$
Wash buffer №2	5,00	1,25
Hemoglobin, mg	0,19 mg	0,1 mg

None of the potentially interfering substances, estimated at the concentrations expected to be detected in normal use, impacts the ability of the kit «Test-BRAF-tissue» to distinguish between mutation-positive and mutation-negative samples of *BRAF* gene.

In addition to the interfering substances study, it was evaluated the impact of necrotic tissue in tumor samples on the ability of «Test-BRAF-tissue» to generate valid data. The study of the impact of necrosis was carried out on 10 samples that had necrosis at a level >50%, as determined by pathology review. After testing using «Test-BRAF-tissue» and interpretation of the results, the obtained data were compared with the results of bidirectional Sanger sequencing. A single result was false-negative due to insufficient DNA amount.

Limitations for testing material use:

- Testing material is not suitable for use if storage and transportation conditions are violated (temperature, duration, multiple freezing and thawing). Testing DNA must be stored at 2 °C to 8 °C and used during 24 hours. It is recommended to store DNA solution at temperature -20 °C for storage more than 24 hours.

- Purity of isolated DNA expressed in ratio to optical dense (A260/280nm) should be no less than 1,4.

- DNA amount sufficient for testing must be not less than 100 ng.

- Samples contaminated with outside biological material are not allowed for use.

- For testing it is necessary to use probes of genomic DNA isolated from histologically proven tumor tissue.

8.3 Storage conditions of testing samples

Storage conditions of the probe of genomic DNA isolated from paraffin-fixed tissue:

Testing DNA must be stored at 2 °C to 8 °C and used during 24 hours. It is recommended to store DNA solution at temperature -20 °C for storage more than 24 hours.

Storage conditions of initial clinical material:

The most available clinical material for DNA isolation is formalin-fixed paraffin-embedded tissue (FFPE-blocks). FFPE-blocks may be stored at room temperature.

Paraffin sections may be stored at room temperature during 4 weeks before DNA isolation.

Storage conditions of the biopsic specimen for DNA isolation:

- at room temperature — during 6 hours;
- at temperature 2–8 °C — during 3 days;
- at temperature minus 20 °C — during 1 week;
- at temperature minus 70 °C — for a long storage.

9. Preparation of the components for testing

Installation, mounting, setup, calibration of the medical product is not required for putting into operation.

Mix thoroughly tubes contents by turning over each tube 10 times or then vortex on low speed during 3-5 seconds. Precipitate drops from tube caps by short centrifugation.

ATTENTION! Do not vortex Taq-polymerase (Taq) because it may inactivate the ferment.

10. Testing procedure

The PCR-testing consists of the following stages:
A) PCR preparation;
B) DNA PCR-amplification and «real-time» hybridization-fluorescent detection of amplification products;
C) interpretation of results (specified in Section 11).

A) PCR preparation

(performed in the pre-amplification area – room for reagents dropping and PCR-amplification)

Total reaction volume – 20 µl.

ATTENTION! It is prohibited to change the reaction volume. If the volume is changed, the method sensitivity will decrease greatly!!!

Before testing it is necessary to prepare reaction mixes (master mixes) for testing DNA, PC and NTC. Mix all necessary components in separate sterile tubes assuming that it is necessary to take 4 µl of PCR mix and 10 µl of Taq-polymerase for one reaction. Use separate tip with an aerosol barrier for each reaction component of each sample.

Prepare master mixes according to Table 5. In the table it is taken into account the stock of reagents (+1 volume of each kind) for compensation of possible losses during pipetting.

ATTENTION! When working with Taq-polymerase take the required volume from the tube while not sinking the tip deeply in the reagent so that not to take excessive enzyme volume because of its getting on the external tip surface.

Table 5 – Preparation of master mixes (calculated for one specimen).

Samples quantity	PCR mix, µl	Taq, µl	Total, µl
1	16	40	56
2	20	50	70
3	24	60	84

4	28	70	98
5	32	80	112
6	36	90	126
7	40	100	140
8	44	110	154
9	48	120	168
10	52	130	182
11	56	140	196
12	60	150	210
13	64	160	224
14	68	170	238
15	72	180	252
16	76	190	266
17	80	200	280
18	84	210	294
19	88	220	308
20	92	230	322
21	96	240	336
22	100	250	350
23	104	260	364
24	108	270	378

1. Put 14 µl of each master mix in the relevant tubes as per the recommended arrangement of reactions (see table 6).

2. Put 6 µl of deionized water in «NTC» tubes.

3. Put 6 µl of PC standard mixture in «PC» tubes.

4. Put 6 µl of DNA samples to «S» tubes.

5. Seal the PCR-plate/close the tubes; make sure that all the covers or the film fit tightly.

6. Remove the PCR-plate/ tubes in order to collect the reaction mixture on the well bottom, while preserving the correct orientation of the plate or tube series.

Table 6 - Recommended arrangement of reactions

96-well plate												
Test	1	2	3	4	5	6	7	8	9	10	11	**12**
V600E/ Ec	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	**S10**
V600K	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	**S10**

S1 – DNA isolated from testing sample №1 etc.

B) DNA PCR-amplification and «real-time» hybridization-fluorescent detection of amplification products

(performed in area for PCR – room for PCR-amplification)

1. Place the tubes into the reaction module of a device for «real-time» PCR. Pay attention to the fact that the devices for «real-time» PCR shall be maintained, calibrated and used in accordance with the manufacturer's recommendations. The use of this kit in a non-calibrated device may affect the test performance.

2. Program the device for fulfillment of a relevant amplification program and fluorescent signal detection as per the description for this device (see tables 7, 8).

Table 7 – Amplification program for devices produced by «DNA-Technology»

Stage	Temperature, °C	Time	Total cycle number
1	95	2 min	1
2	95	5 s	50
3	64	15 s	

Table 8 – Amplification program for other devices

Stage	Temperature, °C	Time	Total cycle number
1	95	2 min	1
2	95	5 s	50
3	62	15 s	

3. Start amplification program with fluorescent signal detection at stage 3.

4. After completing the program, analyze the results.

11. Recording and interpretation of results

Record the results with the use of software of the device used for performing PCR with detection in «real-time» mode. Analyze fluorescent signal accumulation curves by two channels:

– the channel **FAM** records a signal evidencing the accumulation of DNA amplification products of mutant *BRAF* gene variants.

– the channel **HEX** records a signal evidencing the accumulation of DNA amplification products of normal *BRAF* gene variants *(used as internal control sample).*

The results are interpreted based on the presence (or absence) of intersection of the fluorescence curve with a threshold line at the appropriate level that determines the presence (or absence) of the threshold cycle «C_t» value for DNA sample in the corresponding column in the results table.

Interpretation of results in the test samples and control samples is presented in the Table 9 and Table 10.

Table 9 – Interpretation of results in the test samples

Tubes	Mutant DNA of *BRAF* gene is detected	Mutant DNA of *BRAF* gene is not detected	Doubtful result	Invalid result
V600E/Ec, V600K	Channel **FAM:** Ct≤ 35 Channel **HEX:** amplification curve rising and (any Ct) or absence of curve rising.	Channel **FAM:** absence of curve rising. Channel **HEX:** Ct≤ 35	Channel **FAM:** amplification curve rising, Ct > 35 Channel **HEX:** Ct≤ 35	Channel **FAM**: Absence of amplification curve. Channel **HEX**: Absence of amplification curve or Ct> 35.

Table 10 – Interpretation of results in the control samples

Tubes	Correct result	Invalid result
PC	Channel **FAM** and **HEX**: Ct≤ 35	Absence of amplification curve rising at **HEX** and/or **FAM**
NTC	Channel **FAM** and **HEX**: absence of amplification curve rising	Amplification curve rising at channel **FAM** and/or **HEX** (any Ct)

Interpretation of results in control samples

The result of the PCR-testing is considered to be correct if reaction V600E/Ec, V600K are held in tubes with relevant PC not later than 35 cycle and there is absence of amplification curve rising in NTC tubes.

Interpretation of results in test samples

Interpretation of results for test samples is performed only if results for NTC and PC are correct.

The interpretation is performed using software of the device used. Threshold line is set at the level of curves transition into exponential growth phase.

Mutant DNA of *BRAF* gene is detected if amplification curve at FAM channel rises above the established threshold line, and Ct≤35. Channel HEX – amplification curve rising (any Ct) or absence of curve rising.

Mutant DNA of *BRAF* gene is not detected if amplification curve at FAM channel doesn't rise above the established threshold line, and amplification curve at HEX channel rises above the established threshold line, Ct≤ 35 (Internal control sample).

Test result is doubtful if amplification curve at channel FAM rises above established threshold line, and Ct>35. Amplification curve at HEX channel rises above than established threshold line and Ct≤35.

Result is invalid, if amplification curves do not rise at channel FAM. Amplification curve at channel HEX does not rise above established threshold line or rise above established threshold line but at the same time Ct>35.

If an invalid result is obtained for the sample, it is necessary to repeat PCR-testing of the respective test sample starting from DNA isolation from the tissue, or reject the sample as unsuitable for this type of testing.

If a doubtful result is obtained for the sample, it is required to re-peat PCR-testing of the respective test sample starting from DNA isolation from the tissue.

The kit is unsuitable for further use if amplification curves at channels FAM and HEX in PC tubes are lower than the established threshold line and such result is steadily reproduced.

12. Storage, transportation and usage conditions

Storage.

«Test-BRAF-tissue» kit must be stored at 2 °C to 8 °C in the manufacturer packing during all shelf life.

After packing opening kit components should be stored under the following conditions:

- Kit components must be stored at 2 °C to 8 °C during all shelf life;
- PCR V600E/Ec, V600K must be stored in a light-proof place during all shelf life.

Kit stored with violation of storage conditions are not to be applied.

Transportation.

«Test-BRAF-tissue» kit must be transported by all kinds of transport at the covered vehicles in accordance with rules of transportation acting on the transport of this type.

Kit must be transported at temperature from -20 °C to +8 °C during all shelf life. Transportation at room temperature (15–25°C) is acceptable but no longer than 5 days.

Atmosphere pressure is not controlled because it does not influence the sample quality.

For ensuring of transportation conditions during all transportation period the kit is placed into reusable polyurethane-foam thermal container with ice pack for temporary storage and transportation. Type, volume, ice pack amount at transported kits and thermal container volume are selected depending on duration and transportation conditions.

Kits transported with violations of temperature conditions are not to be used.

Shelf life. Shelf life of the kit «Test-BRAF-tissue» is 12 months. The kit shall not be used after the expiry date.

Shelf life of opened kit components. 12 months if stored at 2 °C to 8 °C.

Shelf life of kit components ready for operation. 1 hour if stored in an ice bath and complied with conditions that prevent components drying and contamination by outside biological material.

13. Utilization

Kits that have become unusable including because of the expiration of the expiry date, are subject to utilization in accordance with the requirements of SanPiN 2.1.7.2790-10 «Sanitary and epidemiologic requirements to the address with medical waste».

In accordance with classification of medical waste the kits refer to class A (epidemiologically safe waste approached on structure to municipal solid waste). Unused reagents in accordance with p. 4.28 of SanPiN 2.1.7.2790-10 «Sanitary and epidemiologic requirements to the address with medical waste» are not subject to use gather in the one-time marked packaging of any color (except yellow and red).

Residual tubes and materials are utilized in accordance with MU 287-113 (Methodological instructions for disinfection, pre-sterilization purification and sterilization of medical devices).

Liquid components are eliminated by draining into the sewage system with a preliminary watering of the reagent with tap water 1: 100 and removal of the rest of the packages as industrial or household garbage.

Consumer package of «Test-BRAF-tissue» kit is subject to mechanical destruction with removal of residues as industrial or household garbage.

Personnel carrying out the destruction of the kit must comply with the safety rules for carrying out a particular method of destruction.

14. Warranty obligations, contact information

The manufacturer guarantees the conformity of «Test-BRAF-tissue» kit to technical requirements under transportation, storage and operation conditions established by technical specification.

If there are any complaints regarding the quality, undesired events that may cause adverse event (incident), send the information to the address:

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Tel.: +7 499 705-03-75
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Technical support service:
Tel.: +7 927 981 58 81
E-mail: help@testgen.ru

European Authorized Representative:
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