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INSTRUCTION

Kit for mutations detection in *KRAS* gene based on real-time PCR in the probe of human genomic DNA from paraffin-fixed tissue samples (Test-KRAS-tissue) by TS 21.20.23-006-97638376-2016

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IVD

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1. **Intended use**

Intended use: kit «Test-KRAS-tissue» is intended for professional use in medical centers and clinical diagnostic laboratories of oncological specialization when examining patients with «colorectal cancer» (CRC). It is aimed for qualitative identification of 6 mutations in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and 1 mutation in codon 13 (Gly13Asp) of the *KRAS* gene by real-time allele-specific PCR in human genomic DNA from paraffin-fixed tissue samples for indication of targeted therapy. For this purpose it is used medical drugs based on anti-EGFR monoclonal antibodies in patients diagnosed with «colorectal cancer» with the wild type of the *KRAS* gene.

Application area - clinical laboratory diagnostics, oncology.

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

Determination principle

The testing is carried out by real-time allele-specific PCR. PCR products of the *KRAS* gene are identified in 5′-exonuclease reaction using FAM- and HEX-labeled probes. Kit contains the reagents for detection of 6 mutations in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and 1 mutation in codon 13 (Gly13Asp) of the *KRAS* gene. PCR mixes contain all necessary reagents. Also the kit includes negative control sample (NTC) and positive control sample (PC) containing equimolar mixture of mutations Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys, Gly13Asp in the concentration 5 % and negative control sample (NTC).

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

Target analyte description, information about its scientific validation

Target analyte is *KRAS* gene considered when examining patients with «colorectal cancer» for identification of 6 mutations in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and 1 mutation in codon 13 (Gly13Asp) of the *KRAS* gene for indication of

targeted therapy using medical drugs based on anti-EGFR monoclonal antibodies in patients diagnosed with «colorectal cancer» with the wild type of the *KRAS* gene.

Scientific validation.

Mutations in the *KRAS* gene are often found in human cancers. Such mutations are present in around 30-40% of cases. Now targeted drugs based on monoclonal antibodies - EGFR inhibitors cetuximab (cetuximab) and panitumumab (panitumumab) - are used to treat metastatic CRC. Antibody binding to EGFR leads to inhibition of tumor cells invasion into normal tissues that prevents tumor extension to other organs. Anti-EGFR drugs increase the median survival in patients with the wild type of the *KRAS* gene. Mutations of the *KRAS* gene determine the tumor aggressive-disruptive behavior because CRC grows in the shortest time, metastasizes rapidly and not succumb to chemotherapy. Therefore, it was recommended to determine mutations in the *KRAS* gene among all patients with metastatic CRC.

Currently, targeted drugs based on monoclonal antibodies, EGFR inhibitors cetuximab (cetuximab) and panitumumab (panitumumab), are used to treat metastatic CRC. Antibody binding to EGFR results to the invasion of tumor cells into normal tissues, inhibiting the spread of the tumor to other organs. In patients with the wild-type KRAS gene, anti-EGFR drugs significantly increase median survival. Mutations of the *KRAS* gene determine the aggressive behavior of the tumor: CRC develops in the shortest possible time, quickly metastasizes and answers badly to chemotherapy. Therefore, it was recommended to determine mutations in the *KRAS* gene in all patients with metastatic CRC.

Specific pathology, conditions and risk factor – kit is intended for qualitative identification of 6 mutations in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and 1 mutation in codon 13 (Gly13Asp) of the *KRAS* gene for indication of targeted therapy. For this purpose it is used medical drugs based on anti-EGFR monoclonal antibodies in patients diagnosed with «colorectal cancer» with the wild type of the *KRAS* gene.

Indications and contra indications for use

Indications for use: The kit «Test-KRAS-tissue» is recommended when examining patients with «colorectal cancer» (CRC). It is aimed for qualitative identification of 6 mutations in codon 12 (Gly12Asp,

Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and 1 mutation in codon 13 (Gly13Asp) of the *KRAS* gene by real-time allele-specific PCR in human genomic DNA from paraffin-fixed tissue samples for indication of targeted therapy. For this purpose it is used medical drugs based on anti-EGFR monoclonal antibodies in patients diagnosed with «colorectal cancer» with the wild type of the *KRAS* gene.

Contra indications: none.

The kit is intended for professional use only in medical centers and clinical diagnostic laboratories of oncology specialization. Potential users are doctor of clinical diagnostic laboratory and laboratory technicians.

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

2. Kit characteristics

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

Kit contents

Kit «Test-KRAS-tissue» includes:

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

No.	Reagent	Marking on the tube cap	Description	Tube quantity, volume, µl
1	PCR Mix Gly12Ser	**G12S**	Pink-colored transparent liquid	1 tube (120 µl)
2	PCR Mix Gly12Arg	**G12R**	Pink-colored transparent liquid	1 tube (120 µl)
3	PCR Mix Gly12Cys	**G12C**	Pink-colored transparent liquid	1 tube (120 µl)
4	PCR Mix Gly12Asp	**G12D**	Pink-colored transparent liquid	1 tube (120 µl)
5	PCR Mix Gly12Ala	**G12A**	Pink-colored transparent liquid	1 tube (120 µl)
6	PCR Mix Gly12Val	**G12V**	Pink-colored transparent liquid	1 tube (120 µl)

7	PCR Mix Gly13Asp	**G13D**	Pink-colored transparent liquid	1 tube (120 µl)
8	PC	**К+**	Transparent colorless liquid	1 tube (420 µl)
9	NTC	**К-**	Transparent colorless liquid	1 tube (420 µl)
10	Taq-polymerase	**Taq**	Transparent colorless liquid	2 tubes (1000 µl each)

Positive control (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 *KRAS* gene and artificial-synthesized insertion with 300 bps size in plasmid vector pAL-TA with concentration 20 GE/µl containing equimolar mixture of mutations in 12 codon (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and one mutation in codon 13 (Gly13Asp).

Deionized water is used as NTC.

All reaction mixes contain primers and probes to the internal control sample. Probes to internal control sample are HEX-labeled (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.

3. Operating principle

Qualitative identification of mutations status in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and in codon 13 (Gly13Asp) of the *KRAS* gene by real-time allele-specific PCR in human genomic DNA from paraffin-fixed tissue samples for indication of targeted therapy 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 *KRAS* 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.

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

4 Analytical and diagnostic characteristics

Table 2 – Analytical characteristics of the kit «Test-KRAS-tissue»

Analytical specificity	Specific to mutations in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and one mutation in codon 13 (Gly13Asp) of *KRAS* gene
Analytical sensitivity	10 copies of *KRAS* gene to 1 μl of DNA solution

The list of detected mutations with mutation ID is presented in the Table 3.

Table 3 – the list of detected mutations with specified mutation ID

Mutation	Changes in nucleotids	Amino acids changes	COSMIC ID*
Gly12Asp	c.35G>A	p.G12D	521
Gly12Ala	c.35G>C	p.G12A	522
Gly12Arg	c.34G>C	p.G12R	518

Mutation	Changes in nucleotids	Amino acids changes	COSMIC ID*
Gly12Val	c.35G>T	p.G12V	520
Gly12Ser	c.34G>A	p.G12S	517
Gly12Cys	c.34G>T	p.G12C	516
Gly13Asp	c.38G>A	p.G13D	532

*identification number of mutation according to COSMIC (Catalog of Somatic Mutations in Cancer).

4.2 Diagnostic characteristics:

Diagnostic specificity – 95,0% with 90% confidence interval.

Diagnostic sensitivity – 90,9% with 90% confidence interval.

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.

Limitations

Detection of mutations depends on sample integrity and amount of amplifiable DNA present in the sample. Purity of isolated DNA expressed in ratio of optical dense (A260/280nm) should be no less than 1,4. DNA amount sufficient for testing must be 1-100 ng/μl.

Tumor tissue is not homogenous, so test results may not coincide with results received from other tissue sections of the similar tumor. Besides, tumor samples may include normal tissue (non-tumor tissue). Kit «Test-KRAS-tissue» is not able to detect mutations of *KRAS* gene if genomic DNA is isolated from non-tumor tissue.

During PCR procedure samples may be contaminated. Use caution to avoid contamination of DNA samples and reaction mixtures by PC tube or PCR products.

Kit «Test-KRAS-tissue» may not be used for diagnosis of any disease. It is intended only for identification of mutations status in codon 12 (Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys) and in codon 13 (Gly13Asp) of the *KRAS* gene.

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.

No risks have been identified in the unacceptable zone.

The total residual risk of using the medical product «Kit for mutations detection in *KRAS* gene based on real-time PCR in the probe of human genomic DNA from paraffin-fixed tissue samples (Test-KRAS-tissue) by TS 21.20.23-006-97638376-2016» 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-KRAS-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». Precautions against any special, unusual environmental risks are not provided during use or implementation of the product.

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

Reagents included in «Test-KRAS-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 (e.g., 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 and 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 (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.

8.2 Interfering substances and limitations of sampling material use

For isolation from clinical sample of sufficient DNA amount for PCR-testing and necessary purity, it is recommended to use kits for isolation specified in p. 8.1.

For DNA isolation effectiveness and possible inhibition in the probe that may cause false-negative results, all reaction mixtures contain internal control sample. Internal control is HEX-labeled to distinguish internal control signal from signal of FAM-labeled primers in reactions of *KRAS* gene mutations. Reaction passage is indicative of effective nucleic acids isolation and absence of PCR-inhibitors. Non-reactiveness result should be considered as inaccurate. In this case, it is recommended to make additional DNA isolation for this sample for PCR-testing (see Section 11 «Recording and interpretation of results»).

The effect of potentially interfering substances on the functioning of «Test-KRAS-tissue» was tested for potentially interfering substances that can remain in the human genomic DNA sample after the DNA

isolation, inhibit the PCR reaction and affect the ability of the «Test-KRAS-tissue» to differ between mutant and normal variants of the *KRAS* gene.

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). Potentially interfering substances and their concentrations are shown in Table 4.

Table 4 – Concentration of interfering substances tested during study

Interfering substances	Maximum concentration (µl / 200 µl of DNA solution)	Minimal concentration (µl / 200 µl of DNA solution)
Paraffin (in 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

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

In addition to the interfering substances study, it was evaluated the impact of necrotic tissue in tumor samples on the ability of «Test-KRAS-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-KRAS-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 1-100 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**
Gly12Ser	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly12Arg	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly12Cys	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly12Asp	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly12Ala	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly12Val	NTC	PC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Gly13Asp	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 *KRAS* gene variants.

– the channel **HEX** records a signal evidencing the accumulation of DNA amplification products of normal *KRAS* 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 set.

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 *KRAS* gene is detected	Mutant DNA of *KRAS* gene is not detected	Doubtful result	Invalid result
Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys, Gly13Asp	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	Absence of amplification curve over two channels **HEX** and **FAM**

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 reactions Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys, Gly13Asp 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 *KRAS* 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 *KRAS* 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 neither at channel FAM, nor channel HEX above established threshold line. It indicates that neither normal DNA nor mutant reaction has been tested.

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 repeat 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-KRAS-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 mixes Gly12Asp, Gly12Ala, Gly12Arg, Gly12Val, Gly12Ser, Gly12Cys, Gly13Asp 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-KRAS-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 2 °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-KRAS-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-KRAS-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-KRAS-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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