Instructions for use

Reagent kit for determination of *NRAS* gene mutation status by PCR-RT in human genomic DNA from FFPE tissue samples Test-NRAS-tissue

IVD

Version 3 dated 21.08.2018

Content

1. Intended use

Intended use: Test-NRAS-tissue reagent kit is designed for professional use in medical centers and specialized cancer diagnostic laboratories for examining patients diagnosed with colorectal cancer for quantitative detection of mutations status in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) in *NRAS* gene by real-time allele specific PCR in human genomic DNA extracted from paraffin-fixed tissue samples for targeted therapy indication by anti-EGFR monoclonal antibody drugs in patients diagnosed with colorectal cancer with wild-type *NRAS* gene.

The reagent kit usage area: clinical laboratory diagnostics, oncology.

Test sample type: material for PCR reaction is human genomic DNA from paraffin-fixed tissue samples.

Detection principle

Testing is carried out by real-time allele-specific PCR. Studied *NRAS* gene PCR products are identified in a 5′ exonuclease reaction using FAM- and HEX-labeled probes. The kit contains reagents for mutations determination in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) in *NRAS* gene. PCR mixes contain all the necessary reagents. The kit also contains a positive control sample (PC) including a 5% equimolar mixture of Gly12Asp, Gly12Cys, Gly12Ser, Gly13Asp, Gly13Arg, Gln61Lys, Gln61Leu, Gln61Arg mutations and a negative control sample (NC).

All PCR mixes contain primers and probes for an internal control sample (ICS). ICS probes are HEX-labeled (see Section 11, Results registration and interpretation). It allows to control DNA extraction effectiveness and inhibitors possible presence in the sample that may lead to false negative results.

Target analyte description, information about its scientific validation

Target analyte is *NRAS* gene studied during examination of patients diagnosed with colorectal cancer to qualitatively determine the status of six mutations in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) in *NRAS* gene to determine indications for targeted therapy with anti-EGFR monoclonal antibody drugs in patients diagnosed with colorectal cancer with wild-type *NRAS* gene.

Scientific validity. The Ras family includes H-Ras, K-Ras, N-Ras, R-Ras and other homologous proteins. The Ras signaling pathway reactions cascade sequence acts as a switch that determines gene expression regulation required for a cell to divide or differentiate. Constant Ras activation leads to malignant cell degeneration — point mutations activation. The most well-known oncogenic mutations are mutations in *KRAS* and *NRAS* genes in codons 12, 13 (exon 2), codons 59, 61 (exon 3), codons 117, 146 (exon 4). *KRAS* and *NRAS* mutations lead to constant protein activation and mitotic signal transmission regardless of EGFR stimulation and drug inhibition. This is the reason why therapy with EGFR and tyrosine kinase inhibitors is ineffective in patients with mutated *KRAS* and *NRAS* genes[1].

NRAS gene mutations are associated with colorectal cancer (CRC), melanoma, and multiple myeloma. *NRAS* gene mutations in colorectal cancer account for up to 5%[2].

Nowadays targeted monoclonal antibody drugs — EGFR inhibitors cetuximab and panitumumab, are used for metastatic CRC treatment. Antibody binding to EGFR leads to inhibition of tumor cells invasion into normal tissues preventing tumor spreading to other organs. Anti-EGFR drugs significantly increase median survival in patients with wild-type

[1] Toropova N.E., Zakamova E.V., Teterina Yu.Yu. Molecular and genetic research in practice of oncology clinic/ Toropova N.E., Zakamova E.V., Teterina Yu.Yu. // Proceedings of the Samara Scientific Center of the Russian Academy of Sciences. Agricultural Sciences - 2015. – Vol. 17, - N. 2 (3). - p. 690-696

[2] Vinogradov A. V. Point mutations detection in KRAS and NRAS genes in acute myeloid leukemia using direct automated sequencing

KRAS and *NRAS* genes. Mutations in *KRAS* and *NRAS* genes determine aggressive tumor behavior: CRC develops in the shortest possible time, quickly metastasizes and responds poorly to chemotherapy. Therefore, it was recommended to identify *KRAS* and *NRAS* gene mutations in all patients with metastatic CRC to resolve the anti-EGFR therapy issue.

The in vitro reagent kit is intended for detection, determination and differentiation of a specific pathology, condition or risk factor — the reagent kit is designed for qualitative mutation status determination in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) in *NRAS* gene for examining patients diagnosed with colorectal cancer to determine indications for targeted therapy with anti-EGFR monoclonal antibodies drugs in patients diagnosed with colorectal cancer with wild-type *NRAS* gene.

Indications and contraindications for use

Indications for use: Test-NRAS-tissue reagent kit is designed for examining patients diagnosed with colorectal cancer for qualitative mutation status determination in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) in NRAS gene by real-time allele-specific PCR in human genomic DNA isolated from paraffin-fixed tissue samples to determine indications

Vinogradov A. V., Rezaikin A. V., Sergeev A. G. // Bulletin of Bashkir University - 2014. – Vol. 19, - N. 3. - p. 845-847.

for targeted therapy with anti-EGFR monoclonal antibodies drugs in patients diagnosed with colorectal cancer with wild-type *NRAS* gene.

Contraindications for use: none were identified.

Kit for research use only.

The total time of the procedure is 1 hour.

2. Reagent kit characteristics

Test-NRAS-tissue reagent kit is designed in 1 configuration form. Each kit contains reagents designed for 24 tests.

The reagent kit composition

Test-NRAS-tissue reagent kit consists of:

Table 1 — Test-NRAS-tissue reagent kit composition

No.	Reagent name	Marking on the tube lid	Description	Number of test tubes, volume, µl
1	PCR-mix Gln61Lys	**Q61K**	Transparent pink liquid	1 tube (120 µl)
2	PCR-mix Gly12Asp	**G12D**	Transparent pink liquid	1 tube (120 µl)
3	PCR-mix Gly12Cys	**G12C**	Transparent pink liquid	1 tube (120 µl)
4	PCR-mix Gln61Leu	**Q61L**	Transparent pink liquid	1 tube (120 µl)
5	PCR-mix Gly13Asp	**G13D**	Transparent pink liquid	1 tube (120 µl)
6	PCR-mix Gln61Arg	**Q61R**	Transparent pink liquid	1 tube (120 µl)
7	PCR-mix Gly13Arg	**G13R**	Transparent pink liquid	1 tube (120 µl)
8	PCR-mix Gly12Ser	**G12S**	Transparent pink liquid	1 tube (120 µl)
9	PC	**K+**	Transparent colorless liquid	1 tube (480 µl)

10	NC	**К-**	Transparent colorless liquid	1 tube (480 µl)
11	Taq polymerase	**Taq**	Transparent colorless liquid	2 tubes (1160 µl each)

Positive control sample (PC) is ready to use and is a mixture of Jurkat genomic DNA in 400 *NRAS* gene copies per 1 µl concentration and artificially synthesized 300 bps insertion containing equimolar mixture of *NRAS* gene mutations in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) into plasmid vector pAL-TA in 20 plasmid DNA copies per 1 µl concentration. Contains 5% of mutated and 95% of wild-type DNA copies.

Deionized water is used as an NC.

All PCR mixes contain primers and probes for an internal control sample (ICS). ICS probes are HEX-labeled (see Section 11, Results registration and interpretation). It allows to control DNA extraction effectiveness and inhibitors possible presence in the sample that may lead to false negative results.

3. Method principle

Qualitative status determination of *NRAS* gene mutations in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg) by real-time allele-specific PCR in human genomic DNA isolated from paraffin-fixed tissue samples includes three stages:

1) PCR preparation;
2) DNA PCR amplification with real-time hybridization fluorescence products;
3) results interpretation.

Genomic DNA samples isolated from paraffin-fixed tissue are used for *NRAS* gene regions amplification in a reaction buffer, using primers

specific for these DNA regions and Taq polymerase enzyme. The reaction mixture for amplification contains allele-specific fluorescence labeled oligonucleotide probes that hybridize with a complementary region of the amplified DNA target and get destroyed by Taq polymerase. It leads to fluorescence intensity increase and allows to register specific amplification product accumulation by measuring the fluorescent signal intensity. Fluorescence signal detection is carried out during PCR reaction using a cycler with fluorescence signal detection in «real-time» mode.

Wild-type *NRAS* gene DNA amplification product is detected in the channel corresponding to **HEX** fluorophore, mutated *NRAS* gene DNA amplification product is detected in the channel corresponding to **FAM** fluorophore.

4. The kit analytical and diagnostic characteristics

Table 2 — Test-NRAS-tissue reagent kit analytical characteristics

Analytical specificity	Specific to *NRAS* gene mutations in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg)
Analytical sensitivity	*NRAS* gene 10 copies in 1 µl of DNA solution

A list of identified mutations with mutation ID indication is shown in Table 3.

Table 3 — List of identified mutations with mutation ID indication

A list of mutations differentiated using the Test-NRAS-tissue reagent kit	Nucleotide change	Amino acid change	COSMIC ID*
Gly12Cys	c.34G>T	p.G12C	562
Gly12Ser	c.34G>A	p.G12S	563
Gly12Asp	c.35G>A	p.G12D	564
Gly13Arg	c.37G>C	p.G13R	569
Gly13Asp	c.38G>A	p.G13D	573
Gln61Lys	c.181C>A	p.Q61K	580
Gln61Leu	c.182A>T	p.Q61L	583
Gln61Arg	c.182A>G	p.Q61R	584

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

4.2 Diagnostic characteristics:

Diagnostic specificity is 89.1% with 90% confidence probability

Diagnostic sensitivity is 94.1% with 90% confidence probability

The assay specificity is determined by oligonucleotide primers matched to homologous gene regions as well as by specific fluorescence oligonucleotide probes for hybridization with complementary amplicon regions (specific amplification products). That eliminates cross-reactions.

Method limitations

Mutation detection depends on sample integrity and on amplified DNA amount in a sample. Required for the assay isolated DNA purity expressed in terms of optical densities (A260/280nm) should be at least 1.4. DNA concentration sufficient for the assay should be 1-50 ng/µl.

Tumor tissue is not homogeneous, so the assay results obtained from the tissue sample may not match the results of the same tumor's another section. Also, tumor samples may contain normal (non-tumor tissue). When using a genomic DNA sample isolated from tissue that does not contain a tumor, Test-NRAS-tissue reagent kit will not be able to detect *NRAS* gene mutations.

The PCR method is extremely sensitive to contamination. Be careful to avoid contamination of the test DNA samples and reaction mixtures with the PC tube contents or with PCR products.

Test-NRAS-tissue reagent kit cannot be used for any pathology diagnostics. The reagent kit is designed only for qualitative detection of *NRAS* gene mutations in codon 12 (Gly12Asp, Gly12Cys, Gly12Ser), codon 13 (Gly13Asp, Gly13Arg) and codon 61 (Gln61Lys, Gln61Leu, Gln61Arg).

5. Risks associated with the reagent kit use

Border risk zone includes the following hazards:

- The kit reagents functional properties loss due to transportation, storage or usage under inappropriate conditions;
- The reagent kit disposal in violation of appropriate safety and decontamination measures;
- Test samples cross-contamination;
- Clinical material contamination with inhibiting substances;
- Contamination of reaction mixtures containing DNA test samples with contents from the PC tube or with PCR products;

- Failure to meet the sample preparation, testing procedure and disposal requirements due to unqualified personnel work.

No risks have been identified in the risk zone area.

Total residual risk of using the Test-NRAS-tissue reagent kit for *NRAS* gene mutation status determination by PCR-RT in human genomic DNA from FFPE tissue samples (Test-NRAS-tissue) manufactured by TestGene LLC. The benefit of its usage exceeds the risk.

6. Safety precautions

All components and reagents included in the Test-NRAS-tissue reagent kit belong to low-hazard substances. Precautions against any special, unusual environmental risks when using or selling the product are not provided.

The reagents included in the Test-KRAS-tissue reagent kit have low vapor pressure and exclude the possibility of inhalation poisoning.

The reagents included in the Test-KRAS-tissue reagent kit are non-toxic, as they are prepared by mixing separate non-toxic components.

The work should be carried out in a laboratory performing clinical material molecular-biological (PCR) testing in accordance with sanitary and epidemiological requirements.

The following requirements should always be met when working:

- Remove unused reagents in accordance with sanitary and epidemiological requirements for the management of medical waste.

ATTENTION! When removing waste after amplification (tubes containing PCR products), it is not allowed to open the tubes and spill the contents, as this may lead to contamination of a laboratory area, equipment and reagents with PCR products.

- Use the kit strictly for its intended purpose, according to these instructions;
- Only specially trained personnel are allowed to work with the kit;
- Do not use the kit after the expiration date;

- Avoid contact with skin, eyes and mucous membrane. In case of contact, immediately flush the affected area with water and seek medical assistance.

The necessary precautions are not provided for the magnetic fields effects, external electrical influences, electrostatic discharges, pressure or pressure changes, overloads, or sources of thermal ignition.

The kit contains no substances of human or animal origin with a potential infectious nature, therefore, precautions against any special, unusual risks during product use or sale are not provided.

7. Required equipment and materials

Equipment:

1. PCR-box (e.g. BAV-PCR-Laminar-S, Lamsystems, Russia).
2. Vortex (e.g. TETA-2, Biocom, Russia).
3. A set of electronic or automatic variable volume dispensers (e.g. Eppendorf, Germany).
4. Refrigerator for 2°C... 8°C with a freezer for lower than -16°C.
5. Cycler with real-time fluorescence detection in channels corresponding to the FAM/Green, HEX/Yellow fluorophores: CFX96 (BioRad, USA), DTprime (NPO DNA Technology LLC, Russia), Rotor-Gene Q (Qiagen, Germany), QuantStudio 5 (Thermo Fisher Scientific, USA).

Materials and reagents not included in the kit:

ATTENTION! It is required to use only disposable sterile plastic consumables that have a special “DNase-free” label when working with DNA.

1. Disposable aerosol barrier tips up to 200 µl, 100 µl, 20 µl and 10 µl (e.g., Axygen, USA);
2. Pipette tip racks (e.g. Axygen, USA) and 0.5 (0.2) ml microtube racks (e.g. InterLabService, Russia);

3. Lab coat and disposable talc-free gloves;

4. Container for disinfectant with a lid;

5. Thin-walled disposable PCR 0.2 ml tubes with optically transparent flat lids (if detecting through a lid) or with optically transparent walls (if detecting through a tube wall); or 0.2 ml tube strips or PCR plates with an optically transparent film (e.g. Axygen, USA).

8. Test samples

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

8.1 Obtaining human genomic DNA samples from paraffin-fixed tissue

To isolate necessary for the assay human genomic DNA from formalin-fixed and paraffin-embedded tissues samples it is recommended to use the following reagent kits:

- Reagent kit for human genomic DNA isolation from formalin-fixed and paraffin-embedded tissues (DNA-Tissue-F), manufactured by TestGene LLC, Russia.
- Reagent kit for human genomic DNA isolation from formalin-fixed and paraffin-embedded tissues (DNA-Tissue-M), manufactured by TestGene LLC, Russia.

8.2 Interfering substances and test material use limitations

To isolate from a clinical sample sufficient for PCR assay DNA amount of the required purity, it is recommended to use the isolation kits specified in Section 8.1.

All the PCR mixes contain primers and probes for the internal control sample (ICS) to control the effectiveness of DNA extraction and inhibitors possible presence in the sample, which may lead to obtaining false negative results. ICS probes are HEX-labeled to distinguish the internal control signal from the FAM-labeled primers signals in mutated *NRAS* gene reactions. Reaction passage indicates sufficient nucleic acid extraction efficiency and PCR-inhibitors absence. If there is no reaction

the result should be considered unreliable. In this case it is recommended to re-isolate DNA for PCR-testing (see Section 11, Results registration and interpretation).

The potentially interfering substances effect on the Test-NRAS-tissue reagent kit performance has been examined for potentially interfering substances that may remain in a human genomic DNA sample after DNA isolation, inhibit a PCR reaction and affect the Test-NRAS-tissue reagent kit ability to differentiate *NRAS* gene mutated and wild-type variants.

The potentially interfering substances effect was evaluated by examining the effect of each substance on C_t values and by qualitative determining the mutations status in a test sample in two concentrations (maximum and minimum), which range is expected to be found during a normal use of the Test-NRAS-tissue reagent kit. Potentially interfering substances and their concentrations are shown in Table 4.

Table 4 — Interfering substances concentration examined during interfering substances effect study

Interfering substances	Maximum concentration (µl /200 µl of DNA solution)	Minimum concentration (µl /200 µl of DNA solution)
Paraffin (in xylene)	$2.00*10^{-4}$	$5.00*10^{-5}$
Xylene	$2.00*10^{-4}$	$5.00*10^{-5}$
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 concentrations that are expected to be met during normal use of the Test-NRAS-tissue reagent kit affect the reagent kit ability to differentiate mutated and wild-type variants of the *NRAS* gene.

The impact of necrotic tissue in tumor samples on the Test-NRAS-tissue reagent kit ability to produce reliable results was evaluated in addition to the interfering substances study. The necrosis impact study was conducted using 11 samples that had >50% necrosis according to a pathology review. After testing with the Test-NRAS-tissue reagent kit and results interpretation the obtained data were compared to the Sanger bidirectional sequencing results for the samples. 1 obtained result was false negative due to suspectedly insufficient DNA amount.

Limitations on test material use:

- Test material usage is not allowed under storage and transportation conditions violation (temperature, duration, multiple freezing and thawing). DNA for the assay should be stored at 2°C ... 8°C and be used during 24 hours. To store for more than 24 hours store the DNA mixture at -20°C;
- DNA purity expressed in optical density ratio (A260/280 nm) required for the assay conduction should be at least 1.4;
- DNA concentration sufficient for the assay should be 1-50 ng/µl.
- It is not allowed to use samples contaminated with extraneous biological material;
- It is necessary to use genomic DNA samples isolated from histologically confirmed tumor tissue;

8.3 Test sample storage conditions

Storage conditions for human genomic DNA isolated from paraffin-fixed tissue:

Obtained DNA should be stored at 2°C... 8°C and be used for the assay during 24 hours. To store for more than 24 hours store the DNA mixture at -20°C;

Initial clinical material storage conditions:

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

Paraffin curls may be stored at room temperature for 4 weeks till DNA isolation.

Storage conditions for biopsy material intended for DNA isolation:

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

9. Kit components preparation for testing

The kit does not need to be installed, assembled, adjusted, calibrated for commissioning.

Mix thoroughly the tubes contents by turning each tube 10 times or mix on a vortex at low speed for 3-5 seconds, then remove the drops from the tube lids by a short centrifugation.

10. Testing procedure

PCR testing includes the following steps:

A) PCR preparation;

B) Real-Time DNA PCR amplification with hybridization-fluorescence detection of amplification products;

C) Results interpretation (fully described in Chapter 11).

A) PCR preparation;

(is carried out in the pre-PCR area — a room for reagent dispensing and preparation for PCR amplification)

Total reaction volume is 20 µl.

ATTENTION! It is forbidden to change the reaction volume. If the volume is changed, the method sensitivity decreases dramatically!!!

It is necessary to prepare reaction mixtures (master mixes) for DNA, PC and NC right before the assay. For that mix all the necessary components in separate sterile tubes, 1 reaction requires 4 µl of PCR mix and 10 µl of Taq polymerase. It is necessary to use a separate tip with an aerosol barrier for each reaction component of every sample.

Prepare master mixes according to Table 5. The stock of reagents (+1 volume of each type) is taken into account to compensate for possible losses during pipetting.

ATTENTION! When working with a Taq polymerase take the required volume from the tube not immersing the tip deeply in the reagent to avoid excessive enzyme taking due to its adhering on the external surface of the tip.

Table 5 — Master mix preparation (according to test samples number).

Samples number	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. Add 14 µl of each master mix to the corresponding tubes according to the recommended reactions layout (see Table 6).
2. Add 6 µl of NC into the "NC" tubes.
3. Add 6 µl of PC into the "PC" tubes.
4. Add 6 µl of DNA samples into the "O" tubes.
5. Tap the PCR plate/close the tubes, make sure that all the lids or the film fit tightly.
6. Spin the PCR-plate/tubes on a centrifuge to collect the reaction mixture on the well bottoms while preserving the correct direction of the plate or the tube set.

Table 6 — Recommended reactions layout

96-well plate												
Test	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Gln61Lys	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gly12Asp	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gly12Cys	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gln61Leu	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gly13Asp	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gln61Arg	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gly13Arg	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Gly12Ser	NC	PC	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10

O1 — DNA isolated from test sample No. 1 and etc.

B) PCR DNA amplification and fluorescence hybridization detection of amplification products in real-time mode

(performed in a PCR area — a room for PCR amplification)

1. Load the tubes into a reaction module of a real-time PCR device. The cyclers must be maintained, calibrated and used in accordance with the manufacturer's recommendations. Using the kit in an uncalibrated device may have an impact on the test performance.

2. Program the device to perform the corresponding fluorescence signal amplification and detection programs according to the used cycler instructions. (see Tables 7, 8).

Table 7 — Amplification program for devices manufactured by DNA-Technology

Stage	**Temperature, °C**	**Time**	**Total cycles number**
1	95	2 min	1
2	95	5 sec	50
3	64	15 sec	

ATTENTION! For devices manufactured by DNA-Technology use factory settings of the optical measurements exposure parameters for each channel.

Table 8 — Amplification program for other devices

Stage	**Temperature, °C**	**Time**	**Total cycles number**
1	95	2 min	1
2	95	5 sec	50
3	62	15 sec	

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

4. After the program completion, analyze the results.

11. Results registration and interpretation

Results registration is carried out via the used PCR device software for PCR-testing with detection in "real time" mode. The fluorescent signal accumulation curves are analyzed in two channels:

– signal indicating the DNA amplification products accumulation of *NRAS* gene mutated variants is registered in the **FAM** channel.

– DNA amplification products accumulation of *NRAS* gene wild-type variants *(used as an internal control sample – ICS)* is registered in the **HEX** channel.

The results interpretation is based on whether or not the fluorescence curve crosses the threshold line.

The test samples and control samples results interpretation principle is described in Tables 9 and 10.

ATTENTION! If the CFX 96 cycler is used, it may be necessary to align some graphs with incorrect bias by applying baseline cycles settings (Baseline Threshold → Baseline Cycles).

Table 9 — Test sample results interpretation

Tubes	Mutated *NRAS* gene DNA is detected	Mutated *NRAS* gene DNA is not detected	Doubtful	Invalid
Gly12Asp, Gly12Cys, Gly12Ser, Gly13Asp, Gly13Arg, Gln61Lys, Gln61Leu, Gln61Arg	**FAM** channel: Ct≤ 35 **HEX** channel: amplification curve rise (any Ct) or no amplification curve rise	**FAM** channel: no amplification curve . **HEX** channel: Ct≤ 35	**FAM** channel: amplification curve rise, Ct >35 **HEX** channel: Ct≤ 35	No amplification curve in both **HEX** and **FAM** channels

Table 10 — Control sample results interpretation

Control sample	Detection channel	
	FAM/Green	**HEX/Yellow**
NC	Absent	Ct >35 or absent
PC	Ct ≤35	Ct ≤35

Results interpretation in control samples

If the obtained NC values differ from the ones stated in the Table 10, the entire sample batch results are considered unreliable. In this case, special measures should be taken to eliminate possible contamination.

If the obtained PC values differ from those indicated in Table 10, it is required to repeat amplification of the entire sample batch. If reobtained PC results differ from those indicated in Table 10, the reagents must be replaced.

Results interpretation in test samples

Test sample results interpretation is carried out only if the NC and PC results are correct.

Interpretation is carried out via the used device software. Threshold line is set at the level of curves transition into exponential growth.

Mutated *NRAS* gene DNA is detected if amplification curve in the FAM channel rises above the set threshold line and Ct≤35. There is amplification curve rise (any Ct) or no amplification curve rise in the HEX channel.

Mutated *NRAS* gene DNA is not detected if amplification curve in the FAM channel does not rise above the set threshold line, and amplification curve in the HEX channel rises above the set threshold line, Ct≤ 35 (i.e. passes the ICS).

Test result is doubtful if amplification curve in the FAM channel rises above the set threshold line, and Ct>35. Amplification curve in the HEX channel rises above the set threshold line and Ct≤35.

Test result is invalid if amplification curves do not rise neither in the FAM channel nor in the HEX channel above the set threshold line. It indicates that there were no reactions to neither wild-type nor mutated DNA.

If the probe result was invalid, repeat the PCR-testing of the corresponding sample starting with DNA re-isolation from the tissue sample or reject the sample as unsuitable for the assay.

If the obtained result is doubtful, repeat the PCR-testing of the corresponding sample starting with DNA re-isolation from the tissue sample.

The kit is unusable for further use if amplification curves in the FAM and HEX channels in PC tubes are below the set threshold line and this result is steadily reproduced.

12. Storage, transportation and usage conditions

Storage

Test-NRAS-tissue reagent kit should be stored in the manufacturer's packaging at 2°C...8°C during the entire kit shelf life.

Storage an opened kit under the following conditions:

- kit components must be stored at 2°C... 8°C during the entire shelf life;

- PCR mixes Gly12Asp, Gly12Cys, Gly12Ser, Gly13Asp, Gly13Arg, Gln61Lys, Gln61Leu, Gln61Arg must be stored in a dark place during the entire shelf life.

Reagent kit stored under storage conditions violation cannot be used.

Transportation

Test-NRAS-tissue reagent kit can be transported by all types of covered vehicles in accordance with the transportation rules applicable for the vehicle type.

Transportation is allowed at 2°C... 8°C during the entire shelf-life period. Transportation is allowed at room temperature (15°C... 25°C) up to 5 days.

Atmospheric pressure is not subject to control because it does not affect the product quality.

To ensure compliance with transportation conditions throughout the entire transportation period, the reagent kit should be placed in a reusable polyurethane foam thermal container filled with ice packs for temporary storage and transportation. Ice packs type, volume and their number in a thermal container and the thermal container size varies according to the transportation duration and conditions.

Reagent kits transported under the temperature conditions violation cannot be used.

Shelf Life

Test-NRAS-tissue reagent kit shelf life is 12 months from the acceptance date by the manufacturer’s Quality Control Department (QCD) under all the transportation, storage and usage conditions. A reagent kit with expired shelf life cannot be used.

Opened kit components shelf life is 12 months from the acceptance date by the manufacturer's QCD if stored at 2°C... 8°C.

Ready for use kit components shelf life is 1 hour under conditions that prevent drying of the components as well as contamination by extraneous biological material.

13. Disposal

Reagent kits that have become unusable including the ones with expired shelf life, are subject to disposal in accordance with sanitary and epidemiological requirements for the management of medical waste.

According to medical waste classification the kits belong to Class A (epidemiologically safe waste, which is similar in composition to solid household waste).

Unused reagents are collected in a single-use labeled packaging of any color (except yellow and red) in accordance with sanitary and epidemiological requirements for the management of medical waste.

Used tubes and materials are disposed of in accordance with the requirements for disinfection, pre-sterilization, cleaning and sterilization of medical devices.

Liquid components (reagents, chemical agents) are disposed by draining into a sewer with a reagent preliminary dilution with tap water 1:100 and removing the packages remains as industrial or household garbage.

Test-NRAS-tissue reagent kit consumer packaging is subject to mechanical destruction with the residues removal as industrial or household garbage.

Personnel carrying out the reagent kit destruction must comply with the safety rules for carrying out one or another destruction method.

14. Warranty, contacts

The manufacturer guarantees the Test-BRAF-tissue-multi-24 reagent kit quality and safety during the shelf-life period in compliance with the product transportation and storage requirements, as well as in compliance with the usage rules.

In case of complaints about the reagent kit quality, undesirable events or incidents, submit information to:

Limited Liability Company TestGene (TestGene, LLC),
9, 44 Inzhenerny Proezd, office 13, Ulyanovsk, 432072, Russian Federation
Phone number: +7 (499) 705-03 75
www.testgene.com

Technical Support Service:

Phone number: +7 927 981 58 81
E-mail: help@testgen.ru