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INSTRUCTION FOR USE

Reagent kit for determination of *EGFR* gene mutation status by multiplex PCR-RT in human genomic DNA from FFPE tissue samples “Test-EGFR-tissue-multi”

IVD

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Content

List of abbreviations

Abbreviations and designations used in the instruction:

PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
ICS	internal control sample
PC	positive control sample
SC	specificity control sample
ID	identification number
COSMIC	Catalog of Somatic Mutations in Cancer
FFPE-block	Formalin-Fixed Paraffin-Embedded tissue
TK	tyrosine kinase
NSCLC	non-small cells lung cancer

Introduction

Lung cancer is one of the most common types of malignant cancers. About 60 thousand people die of lung cancer in Russia every year, which is 20% of deaths from malignant tumors. Histopathologically lung cancer is divided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). In most patients pathology is diagnosed at late stages and has a poor prognosis with an overall 10-15% survival rate in 5 years. 85-90% of all cancer cases are NSCLC.[1]

The Epidermal Growth Factor Receptor (EGFR) mechanism identification and interpretation became one of the most promising discoveries in oncology. EGFR overexpression high frequency has been found to be common for non-small cell lung cancer (40 - 70%), ovarian cancer (35 - 70%), colorectal cancer (25 - 77%) and others. Increased *EGFR* gene expression detection is a negative prognosis factor of the disease course. Activation of mechanisms involving the *EGFR* gene leads to tumor cells proliferative activity increase, neoangiogenesis, delayed apoptosis, and metastases earlier appearance.

Target analyte detected with the Test-EGFR-tissue-multi reagent kit is mutations in the *EGFR* gene — G719S, G719C, G719D, G719A in exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them) and L861Q in exon 21.

[1] Schneider O.V., Kamilova T.A., Golota A.S., Sarana A.M., Shcherbak S.G. Biomarkers and Target Therapy for Lung Cancer // Physical and rehabilitation medicine medical rehabilitation — 2021. — 3 (1).

Target analyte scientific validity lies in its specificity (unique DNA sequence) against the *EGFR* gene somatic mutations found in patients with non-small cell lung cancer (NSCLC), localized in exons 18-21, encoding the tyrosine kinase domain.

According to the clinical guidelines "Malignant neoplasm of the bronchi and lung" (approved by the Ministry of Health of the Russian Federation 2021):

“During molecular genetic studies it is recommended to analyze the EGFR gene mutations for common genetic disorders (deletions in exon 19; p.L858R point substitution in exon 21) as well as for less common genetic disorders in exons 18-21 (including insertions in exon 19 as well as p.L861Q, p.G719X, p.S768I point substitutions).”

Mutations presence in the *EGFR* gene makes signaling pathway activation independent of the ligand presence and provokes cell division, survival and anti-apoptotic signaling. The *EGFR* gene inhibition causes pro-apoptotic molecules upregulation, which ultimately promotes the intrinsic mitochondrial apoptotic signaling pathway activation.[2]

The most frequent and studied mutations are:

L858R — leucine substitution by arginine substitution in codon 858 exon 21 (41%) and deletions in exon 19 (45% of all mutations).

G719X is a point mutation that substitutes glycine at position 719 with other residues such as alanine, cysteine, and serine (occurrence about 3%).[3]

L861Q mutation is also a point mutation in the *EGFR* gene with occurrence about 2%. Insertions occurrence in exon 20 is up to 12%. These mutations can cause ligand-independent pathway activation and initiate carcinogenesis.

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[2] Nasretdinov A.F., Sultanbaev A.V., Menshikov K.V. and others. Landscape of epidermal growth factor gene mutations in lung cancer patients in the Republic of Bashkortostan // Effective pharmacotherapy. – 2020. – Vol. 16. – №33. – P. 18-23.

[3] Li K., Yang M., Liang N., Li S. Determining EGFR-TKI sensitivity of G719X and other uncommon EGFR mutations in non-small cell lung cancer: perplexity and solution (review) // Oncol. Rep. – 2017. – Vol. 37. – № 3. – P. 1347-1358.

The rarest point mutation is S768I, occurs in 0.59-1.9% cases. [4]

Tyrosine kinase (TK) inhibitors are prescribed to patients with NSCLC with mutations in the *EGFR* gene exons 18-21, associated with sensitivity to tyrosine kinase inhibitors (TK). This is an effective treatment method that leads to a significant reduction in the tumor foci size and allows to control disease manifestations for a sufficiently long period of time.

T790M mutation is associated with resistance to tyrosine kinase (TK) inhibitors and is responsible for resistance to TK inhibitor therapy. T790M mutation detection allows to optimize treatment tactics, identify tumors that are not sensitive to the current therapy, and timely prescribe another chemotherapy regimen to a patient. It is clinically and economically beneficial taking into account the long-term use and the therapy high cost.[5]

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

Indications and contraindications for use

Indications for use: Test-EGFR-tissue-multi reagent kit is recommended for examination of patients diagnosed with non-small cell lung cancer stage IB-IIIA and stage IV to qualitatively detect *EGFR* gene G719S, G719C, G719D, G719A mutations status in exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them) S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them) and L861Q mutation in exon 21 by multiplex PCR-RT in human genomic DNA from FFPE tissue samples to determine indications for targeting therapy with small molecular *EGFR* tyrosine kinase inhibitors.

Contraindications for use: none were identified if used by well-trained personnel and taken into account the intended use.

[4] Leventakos K., Kipp B.R., Rumilla K.M. et al. S768I mutation in EGFR in patients with lung cancer // J. Thorac. Oncol. – 2016. – Vol. 11. – № 10. – P. 1798-1801.

[5] Real-time PCR and digital PCR approach for detecting EGFR status in plasma of patients with NSCLC / M. Gordiev [et al.] // Journal of Thoracic Oncology. - 2016. - V 11. - N 4. - p. 124-125. doi:10.1016/S1556-0864(16)30263-5.

Population and demographic aspects of the reagent kit usage: no population or demographic usage aspects of the Test-EGFR-tissue-multi reagent kit were identified.

Sterility: the kit is not sterile.

1. Intended use

Intended use: Test-EGFR-tissue-multi reagent kit is designed for qualitative determination of the *EGFR* gene mutation status — G719S, G719C, G719D, G719A in exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them) and L861Q in exon 21 by multiplex PCR-RT with hybridization-fluorescence detection in human DNA isolated from tissue samples, fixed in 10% formalin solution and embedded in a paraffin block (FFPE-block) when examining patients diagnosed with non-small cell lung cancer (NSCLC) of IB–IIIA and IV stages in order to determine an effective strategy for targeted therapy by low molecular weight EGFR tyrosine kinase inhibitors.

Functional use: Obtained results can be used for patients diagnosed with non-small cell lung cancer of IB–IIIA and IV stages examination in order to determine an effective strategy for targeted therapy by low molecular weight EGFR tyrosine kinase inhibitors.

Reagent kit potential consumers:

Kit for research use only.

2. Method Principle

Method

Real-time multiplex polymerase chain reaction with hybridization-fluorescence detection.

Test sample type

Test material for PCR is DNA samples isolated from tissue fixed in 10% formalin solution and embedded in a paraffin block (FFPE-block).

Detection principle

The *EGFR* gene mutations status determination - G719S, G719C, G719D, G719A in exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R

(detects 2 mutations but does not differentiate them) and L861Q in exon 21 by real-time multiplex polymerase chain reaction with hybridization-fluorescence detection in a genomic DNA sample isolated from biomaterial includes three stages:

1. PCR preparation;
2. DNA PCR-RT amplification with hybridization-fluorescence detection of amplification products;
3. Results interpretation.

DNA samples are used for conducting genomic DNA sections amplification reactions using specific to them primers in a reaction buffer.

PCR Buffer contains all the main reagents including a thermostable hot-start DNA polymerase, dNTP mixture, uracil-DNA glycosidase and an optimized buffer. Uracil-DNA glycosylase enzyme prevents obtaining false positive results during amplification products contamination. Enzyme gets completely inactivated during the first DNA denaturation cycle and does not interfere with the current reaction products amplification.

The primer-mix contains fluorescent labeled oligonucleotide probes that hybridize with an amplified target DNA complementary region and gets destroyed by *Taq* polymerase. The dye and quencher separate, and fluorescence intensity increases. It allows to record the specific amplification product accumulation by measuring the fluorescent signal intensity in real time.

The kit contains reagents for G719S, G719C, G719D, G719A mutations detection in the *EGFR* gene exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations, but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them), and L861Q in exon 21 as well as *EGFR* wild-type gene used as sample volume control (SVC) (Table 1).

Table 1 - The reagent kit multiplexes composition

Multiplex (primer-mix)	**A channel corresponding to a fluorophore**	
	FAM/Green	**HEX/Yellow**
719	G719S, G719C, G719D, G719A mutations in the *EGFR* gene exon 18 (detects mutations but does not differentiate them)	SVC (human *EGFR* gene)
768	S768I mutation in the *EGFR* gene exon 20	SVC (human *EGFR* gene)
790	T790M mutation in the *EGFR* gene exon 20)	SVC (human *EGFR* gene)
858	L858R mutation in the *EGFR* gene exon 21 (detects 2 mutations but does not differentiate them)	SVC (human *EGFR* gene)
861	L861Q mutation in the *EGFR* gene exon 21	SVC (human *EGFR* gene)
Del	Deletions in the *EGFR* gene exon 19 (detects 35 mutations but does not differentiate them)	SVC (human *EGFR* gene)
Ins	Insertions in the *EGFR* gene exon 20 (detects 4 mutations but does not differentiate them)	SVC (human *EGFR* gene)

SVC allows to confirm biomaterial sampling, to assess DNA extraction quality, efficiency and possible inhibitors presence in the sample, which may lead to false negative results.

Method limitations

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

The tumor tissue is not homogeneous, so the analysis 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-EGFR-tissue-multi reagent kit will not be able to detect mutations in the *EGFR* gene.

A possible reason for obtaining a false positive result is contamination during DNA extraction or during multiplex PCR reaction stages. A false positive result can be detected with a negative control sample.

Test-EGFR-tissue-multi reagent kit cannot be used for any pathology diagnostics. The reagent kit is designed only for G719S, G719C, G719D, G719A mutations status qualitative detection in the *EGFR* gene exon 18 (detects G719S, G719C, G719D, G719A mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them) and L861Q in exon 21 in the *EGFR* gene.

The reagent kit package violation during transportation.

The kit usage after the expiration date or after the storage conditions violation.

The reagent kit package violation during transportation.

The mutiplex PCR analysis takes about 60 minutes (time for sample preparation is not included), the exact time depends on the used cycler type.

3. Reagent kit components

Complectation

Test-EGFR-tissue-multi reagent kit comes in 1 configuration.

Test samples number

The reagent kit is designed for 30 reactions of each multiplex (719 — G719S, G719C, G719D, G719A mutations; 768 — S768I; 790 — T790M; 858 — L858R (2 mutations); 861 — L861Q; del — 35 deletions and ins — 4 insertions), that corresponds to 24 test samples with NC and PC in each run or 10 single runs with NC and PC.

Reagent kit components

Table 2 – Test-EGFR-tissue-multi reagent kit components

No	Reagent	Short name on the kid tubes	Description	Quantity, Volume
1	PCR buffer	PCR-b	Transparent colorless liquid	1 tube, 840 µl
2	Primer-mix 719	719	Transparent colorless liquid, may have a lilac shade	1 tube, 300 µl
3	Primer-mix 768	768	Transparent colorless liquid, may have a lilac shade	1 tube, 300 µl
4	Primer-mix 790	790	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl
5	Primer-mix 858	858	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl
6	Primer-mix 861	861	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl
7	Primer-mix del	del	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl
8	Primer-mix ins	ins	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl
9	PC	PC	Transparent colorless liquid	1 tube 420 µl
10	NC	NC	Transparent colorless liquid	1 tube 420 µl

NOTE: operational documentation (instructions for use and quality certificate) is not included in the product, but is included in the product delivery set. To ensure compliance with transportation conditions the reagent kit is placed in a reusable polyurethane foam thermal container filled with ice packs for temporary storage and transportation. The thermal container is put into a cardboard box with the instruction for use and the quality certificate for every reagent kit batch.

PCR Buffer is ready to use and contains all the necessary reagents including thermostable hot-start DNA polymerase, deoxynucleotide triphosphates (including deoxyuridine triphosphate), uracil-DNA glycosylase (UDG), ROX reference dye: $MgCl_2$ and an optimized PCR buffer.

Primer-mix 719 is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for G719S, G719C, G719D mutations (detects mutations but does not differentiate them) in the *EGFR* gene exon 18 (detection in the FAM/Green channel) and for the *EGFR* wild-type gene, used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix 768 is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the S768I mutation in the *EGFR* gene (detection is carried out in the FAM/Green channel) and for the *EGFR* wild-type gene, used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix 790 is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the T790M mutation in the *EGFR* gene exon 20 (detection is carried out in the FAM/Green channel) and for the *EGFR* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix 858 is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the L858R mutation in the *EGFR* gene exon 21 (detects 2 mutations but does not differentiate them) (detection is carried out in the FAM/Green channel) and for the *EGFR* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix 861 is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the L861Q mutation in the *EGFR* gene exon 21 (detection is carried out in the FAM/Green channel) and for the *EGFR* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix del is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes

for 35 mutations in the *EGFR* gene exon 19 (detects mutations but does not differentiate them) (detection is carried out in the FAM/Green channel) and for the *EGFR* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Primer-mix ins is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for 4 mutations in the *EGFR* gene exon 20 (detects mutations but does not differentiate them) (detection is carried out in the FAM/Green channel) and for *EGFR* wild-type gene used as sampling volume control (SVC) (detection is carried out via the HEX/Yellow channel).

Positive control sample (PC) is ready to use and is a plasmid DNA mixture with 12.5% of mutated and 87.5% of wild-type gene copies.

Negative control sample (NC) is ready to use and is DNase-free deionized water.

The kit contains no substances for medical use, substances of human or animal origin.

4. Reagent kit characteristics

4.1 Technical and functional characteristics

Table 3 — Test-EGFR-tissue-multi reagent kit components

Indicator name		**Characteristics and standards**		**Clause in technical Specification (TS)**
1.1 Technical characteristics				
Reagent	**Short name on a tube lid**	**Description**	**Quality, volume, µl (±5%)**	
PCR-buffer	PCR-b	Transparent colorless liquid	1 tube 840 µl	Section 7, clause 7.6
Primer-mix 719	719	Transparent colorless liquid may have a lilac shade	1 tube 300 µl	Section 7, clause 7.6
Primer-mix 768	768	Transparent colorless liquid, may have a lilac shade	1 tube, 300 µl	Section 7, clause 7.6
Primer-mix 790	790	Transparent colorless liquid, may have a lilac shade	1 tube, 300 µl	Section 7, clause 7.6
Primer-mix 858	858	Transparent colorless liquid, may have a lilac shade	1 tube, 300 µl	Section 7, clause 7.6

Prime-mix 861	861	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl	Section 7, clause 7.6
Primer-mix del	del	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl	Section 7, clause 7.6
Primer-mix ins	ins	Transparent colorless liquid, may have a lilac shade	1 tube 300 µl	Section 7, clause 7.6
Indicator name		**Characteristics and standards**		**Clause in Technical Specification (TS)**
PC	PC	Transparent colorless liquid	1 tube, 420 µl	Section 7, clause 7.6
NC	NC	Transparent colorless liquid	1 tube 420 µl	Section 7, clause 7.6
1.2 Completeness		Clause 1.4		Section 7, clause 7.10
1.3. Marking		Clause 4		Section 7, clause 7.10
1.4. Package		Clause 5		Section 7, clause 7.10
2. Functional characteristics				
2.1. Positive result with PC		Fluorescence signal growth recorded in tubes with PC in the FAM/Green Ct≤35 and HEX/Yellow Ct≤35		Section 7, clause 7.8.2
2.2. Negative result with NC		In tubes with NC in the FAM/Green, HEX/Yellow channels Ct is not indicated (i.e. no fluorescence accumulation graph).		Section 7, clause 7.8.2
2.3. Reaction in tubes with SC		In tubes with SC in the FAM/Green channel Ct is not indicated (i.e. no fluorescence accumulation graph) or in the del multiplex Ct ≥35 and in the HEX channel Ct≤35.		Section 7, clause 7.8.2

NOTE: human genomic DNA mixture isolated from the U-937 cell line in 1 000 copies per 1 ml concentration is used as a specificity control sample (SC) during a control PCR.

In case of the reagent kit failure, functional deviations that may affect the kit safety or the kit analytical characteristics immediately stop using the kit and inform the manufacturer (see section 14 of the instruction).

The control sample metrological traceability

The control sample metrological traceability is confirmed via the spectrometry method by checking the U-937 stock solution concentration (wild-type gene DNA copies) and plasmid mutations concentration in the *EGFR* gene: G719S, G719C, G719D, G719A, S768I, T790M, L858R, L861Q, Del and Ins gene region — mutant DNA copies in control sample metrological traceability.

4.2 Analytical efficiency characteristics

4.2.1 Analytical specificity

Specific to G719S, G719C, G719D, G719A mutations in exon 18 (detects mutations but does not differentiate them), deletions in exon 19 (detects 35 mutations but does not differentiate them), S768I, T790M and insertions in exon 20 (detects 4 mutations but does not differentiate them), L858R (detects 2 mutations but does not differentiate them), and L861Q in exon 21 of the *EGFR* gene and to the human *EGFR* wild-type gene.

EGFR gene target regions analytical specificity was approved *in silico* via the BLAST Resource *(https://blast.ncbi.nlm.nih.gov/Blast.cgi).*

A list of differentiated mutations with the COSMIC ID* mutation identification is shown in Table 5 (the reagent kit detects each of the 35 deletions and 4 insertions presence but does not differentiate them).

Table 5 - List of differentiated mutations with ID* indication

A list of mutations differentiated using the Test-BRAF *EGFR*-tissue-Multi-24 reagent kit			COSMIC ID*
Exon 18	G719S	2155 G>A	6252
	G719C	2155 G>T	6239
	G719D	2156 G>A	6252
	G719A	2156 G>C	6239
Exon 19	Del	2235_2249del15	6223
		2235_2252>AAT (complex)	13551
		2236_2253del18	12728
		2237_2251del15	12678
		2237_2254del18	12367
		2237_2255>T (complex)	12384
		2236_2250del15	6225
		2238_2255del18	6220
		2238_2248>GC (complex)	12422
		2238_2252>GCA (complex)	12419
		2239_2247del9	6218
		2239_2253del15	6254

		2239_2256del18	6255
		2239_2248TTAAGAGAAG>C	12382
		2239_2258>CA (complex)	12387
		2240_2251del12	6210
		2240_2257del18	12370
			12369
		2239_2251>C (complex)	12383
		2238_2252del15	23571
		2237_2252>T (complex)	12386
		2236_2252>AT (complex)	26680
		2236_2251>T (complex)	26513
		2235_2255>GGT (complex)	85797
		c.2235_2246del12	28517
		2235_2251>AG (complex)	13549
		2236_2253>CAA (complex)	22999
		2237_2257>TCT	18427
		2233_2247>del15	26038
		2235_2255>AAT	12385
		2239_2256>CAA	12403
		2237_2253>TTGCT	12416
		2235_2248>AATTC	13550
		2235_2251>AATTC	13552
		2253_2276>del24	13556
Exon 20	S768I	2303 G>T	6241
	T790M	2369 C>T	6240
	ins	2307_2308ins9GCCAGCGTG	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
		2309_2310AC>CCAGCGTGGAT	13558
Exon 21	L858R	2573T>G	6224
		2573_2574TG>GT	12429
	L861Q	2582T>A	6213

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

4.2.2 Analytical sensitivity

10 copies of the *EGFR* gene in 1 µl of DNA solution.

4.2.3 Accuracy under repeatability conditions

To assess accuracy under repeatability conditions PC, NC, SC were examined in 10 repetitions.

Repeatability data are obtained within one laboratory for specific equipment and within a specific reagent kit batch.

To precise the accuracy under repeatability the sample arithmetic mean, dispersion, standard deviation, and variation index coefficient are calculated based on the data obtained in control samples repetitions.

Essay results showed that the variation index under repeatability is not higher than 3%.

4.2.4. Accuracy under reproducibility conditions

The test system reproducibility assessment is carried out similarly to the accuracy under repeatability conditions calculation. However, different batches of the reagent kit are used for testing and testings are carried out in different laboratories, by different operators, on different days, via different PCR cyclers (Reproducibility test Block 1, Reproducibility test Block 2, Reproducibility test Block 3, Reproducibility test Block 4).

When conducting accuracy testing under reproducibility conditions variation index was not higher than 5%.

4.2.5 The minimum tumor amount for analysis is 20% according to the results of tumor tissue morphological examination by a histotechnologist.

4.2.6 Detection limit (LoD), as the declared alleles lowest frequency in a sample in the *EGFR* genes, that is able to detect the product — 5%.

4.2.7 The product-specificity in relation to skin microflora has been confirmed in the highest pathogen concentration. No nonspecific reactions were detected during ATCC strains examination (American Type Culture Collection, USA): *Streptococcus pneumoniae* (ATCC® 49619™), *Staphylococcus aureus* subsp. *aureus*, Strain Seattle 1945 (ATCC® 25923™), *Klebsiella pneumoniue* (ATCC® BAA-1705™), *Mycoplasma pneumoniae*, Strain PI 1428 (ATCC® 29085™), *Chlamydophila pneumoniae*, Strain CM-1 (ATCC® VR-1360™), *Legionella pneumophila* subsp. *pneumophila*, Strain Philadelphia 1 (ATCC® 33152™), *Staphylococcus epidermidis*, FDA Strain PCI 1200 (ATCC® 12228™) in concentration from 10^6 to 10^7 cells per ml. The above-mentioned microorganisms do not affect the Test-EGFR-tissue-multi reagent kit ability to differentiate the *EGFR* gene mutated and wild-type variants.

4.2.8. The kit was tested on the *ERBB2/HER2, ERBB3/HER3, ERBB4/HER4* homologous genes. Test-EGFR-tissue-multi reagent kit

does not cross-react with *ERBB2/HER2*, *ERBB3/HER3*, *ERBB4/HER4* homologous genes.

4.2.9 Interfering substances effect

Assay results on the interfering substances effects evaluation are described in Section 8.3 of the instruction.

4.3. Clinical Effectiveness

105 tissue samples fixed in 10% formalin solution and embedded in a paraffin block (FFPE-block) of patients diagnosed with non-small cell lung cancer of IB–IIIA and IV stages with confirmed positive status of the examined mutations in the EGFR gene were used for clinical essay conduction.

Each sample was tested in two series by the Test-EGFR-tissue-multi reagent kit manufactured by TestGene, LLC.

Cyclers recommended by the reagent kit manufacturer that were used for PCR testing:

- DTprime Detecting Cycler (DNA-Technology, LLC, Russia);
- CFX 96 Cycler (Bio-Rad, USA);
- Rotor-Gene Q Cycler (Qiagen, Germany);
- QuantStudio 5 Cycler (Thermo Fisher Scientific, USA),

Results reproducibility is 100%. The reagent kit diagnostic characteristics study results in relation to each detected mutation in the *EGFR* gene are shown in the Table 6.

Table 6

Analyte to be analyzed	Mutation	**Positive samples observation number**	**Negative samples observation number**	**Diagnostic sensitivity** with 95% confidence probability	**Diagnostic specificity** with 95% confidence probability
G719S, G719C, G719D, G719A mutations in the EGFR gene exon 18 (detects mutations but does not differentiate them)	G719S (2155G>A)	8	202	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 99,55%-100%)
	G719C (2155 G>T)	2	208		
	G719D (2156 G>A)	2	208		
	G719A (2156 G>C)	2	208		
	2235_2249del15	10	200		
	2235_2252>AAT				

Deletions in the *EGFR* gene exon 19 (detects 35 mutations but does not differentiate them)	(complex)	2	208	100% (95% diagnostic interval: 97,11%-100%)	100% (95% diagnostic interval: 99,95%-100%)
	2236_2253del18	2	208		
	2237_2251del15	6	204		
	2237_2254del18	2	208		
	2237_2255>T (complex) (E746_S752>V)	6	204		
	2236_2250del15	8	202		
	2238_2255del18	10	200		
	2238_2248>GC (complex) (L747_A750>P)	2	208		
	2238_2252>GCA (complex)	2	208		
	2239_2247del9	2	208		
	2239_2253del15	2	208		
	2239_2256del18	8	202		
	2239_2248TTAAGAGAAG>C	2	208		
	2239_2258>CA (complex) (L747_P753>Q)	6	204		
	2240_2251del12	8	202		
	2240_2257del18	2	208		
	2240_2254del15	4	206		
	2239_2251>C (complex) (L747_T751>P)	6	204		
	2238_2252del15	2	208		
	2237_2252>T (complex)	4	206		
	2236_2252>AT (complex)	2	208		
	2236_2251>T (complex)	2	208		
	2235_2255>GGT (complex)	2	208		
	c.2235_2246del12	4	206		
	2235_2251>AG (complex)	2	208		
	2236_2253>CAA (complex)	2	208		
	2237_2257>TCT	2	208		
	2233_2247>del15	2	208		

	2235_2255>AAT	2	208		
	2239_2256>CAA	2	208		
	2237_2253>TTGCT	2	208		
	2235_2248>AATTC	2	208		
	2235_2251>AATTC	2	208		
	2253_2276>del24	2	208		
S768I mutation in the *EGFR* gene exon 20	S768I (2303 G>T)	2	208	100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 98,24%-100%)
T790M mutation in the EGFR gene *exon 20*	T790M (2369C>T)	14	196	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 98,14%-100%)
Insertions in the *EGFR* gene exon 20 (detects 4 mutations but does not differentiate them)	2307_2308ins9GCCAGCGTG	2	208	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 99,55%-100%)
	2319_2320insCAC	2	208		
	2310_2311insGGT	6	204		
	2309_2310AC>CCAGCGTGGAT	4	206		
L858R mutation in the *EGFR* gene exon 21 (detects 2 mutations but does not differentiate them)	L858R(2573T>G)	36	174	100% (95% diagnostic interval: 90,75%-100%)	100% (95% diagnostic interval: 99,04%-100%)
	L858R(2573_2574TG>GT)	2	208		
L861Q mutation in the EGFR gene *exon 21*	L861Q (2582T>A)	2	208	100% (95% diagnostic interval: 15,81%-100%)	100% (95% diagnostic interval: 98,24%-100%)

5. Risks associated with the reagent kit usage

The risk zone includes the following hazards:

1. The kit reagents functional properties loss due to transportation, storage or usage under inappropriate conditions;
2. Test samples cross-contamination;
3. Clinical material contamination with inhibiting substances in concentrations exceeding the permissible ones;
4. Reaction mixtures and test DNA samples contamination with contents from a PC tube or with amplification products;
5. Testing with a poor-quality DNA sample (low concentration and/or poor purification);
6. Failure to comply with the requirements for sample preparation, analysis and disposal due to unqualified personnel work;
7. Usage of an unusable kit (after the expiration date or in case of damaged package).

No risks have been identified in the risk zone area.

Total residual risk of using the Test-EGFR-tissue-multi reagent kit for *EGFR* gene mutation qualitative status determination by multiplex polymerase chain reaction in real-time in human genomic DNA from FFPE tissue samples is acceptable; the benefit of its usage exceeds the risk.

6. Safety precautions

All components and reagents included in Test-EGFR-tissue-multi 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-EGFR-tissue-multi reagent kit have low vapor pressure and exclude the possibility of inhalation poisoning.

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

Personnel should ensure and comply with the biological safety rules and work requirements for the organization and conduct it in order to prevent contamination with nucleic acids and (or) amplicons of the tested samples, premises and equipment.

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.

1. use the kit strictly for its intended purpose, according to this instruction;
2. do not use the kit if the package is violated;
3. only specially trained personnel are allowed to work with the kit (a specialist with higher medical education who has been trained in licensed qualification courses to conduct PCR testing, as well as a laboratory assistant with secondary special medical education);
4. do not use the kit after the expiration date;
5. 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

Work with the reagent kit is carried out in working area 3 (for preparing reactions)

Multiplex PCR equipment:

1. Class II and III biological safety cabinet;
2. Vortex.
3. A set of electronic or automatic variable volume dispensers;
4. Refrigerator for 2°C... 8°C with a freezer for less than -16°C;
5. Cycler[6] with real-time fluorescence detection in the channels corresponding to the FAM/Green and HEX/Yellow fluorophores:

- DTprime (DNA-Technology LLC, Russian Federation, registration certificate № FSR 2011/10229 dated 03.03.2011);

- CFX 96 (BioRad, USA, registration certificate № FSZ 2008/03399 dated 21.06.2016);

- Rotor-Gene Q (Qiagen, Germany, registration certificate № FSZ 2010/07595 dated 10.08.2010);

- QuantStudio 5 (Thermo Fisher Scientific, USA, registration certificate № RZN 2019/8446 dated 06.06.2019).

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.

- Disposal tips with an aerosol barrier up to 1,000 µl, 200 µl, 20 µl and 10 µl (e.g., Axygen, USA);

- 1.5ml Disposal Eppendorf type sterile tubes;

- PCR plates with an optically transparent film (e.g., Axygen, USA) or thin-walled disposable PCR test tubes with an optically transparent lid:

- 0.2ml PCR tubes (with optically transparent walls if the detection is carried out through a tube wall),

- 0.2ml PCR strip tubes;

- Lab coat and disposable talc-free gloves; Container with disinfectant;

- Test tube rack for 0.2ml tubes or for 0.2ml tube strips (e.g., InterLabService, Russian Federation).

7. Reagent kit for DNA extraction from tissue samples, fixed in 10% formalin solution and embedded in a paraffin block — FFPE-block (see section 8.2 of the instruction).

[6] The cyclers must be maintained, calibrated and used in accordance with the manufacturer's recommendations. The kit usage in an uncalibrated device may have an impact on the performance of the test.

8. Test samples

Test sample type

PCR test material is human genomic DNA samples, fixed in 10% formalin solution and paraffin-embedded tissue (FFPE-block).

8.1 Clinical material collection procedure

Biological material should be collected and packed by healthcare providers specially trained to follow biological safety requirements and rules when working and collecting material suspected of being infected with microorganisms of pathogenicity group I-IV.

Material collection for testing

Biopsy and/or surgical specimen.

The material is sampled from a pathological lesion: from its central part and from the part bordering with unchanged tissues. The sampled material is placed in a container with 10% neutral formalin solution. After fixation, laboratory processing of biological material is performed, which includes the following procedures — impregnation (dehydration and impregnation with paraffin); embedding in paraffin and paraffin blocks preparation — FFPE-blocks); microtomy (paraffin sectioning).

Histological preparations suitability criteria for DNA isolation for tumor cells subsequent molecular genetic analysis:

1. According to the morphological analysis results tumor zones should occupy at least 60% of the tissue in a FFPE slide;
2. According to the morphological analysis results hemorrhage and necrosis areas should occupy not more than 15% of the tissue in a FFPE slide;

If the sample does not meet at least one of the listed criteria, it is recommended to use another sample.

When preparing paraffin slides, it is necessary to minimize the samples cross-contamination risk. For that:

- work in disposable talc-free gloves;
- perform the procedure in a PCR cabinet or in a laminar flow cabinet;
- use disposable microtome blades and sterile tweezers;
- dispose the first two slides of each block, for molecular research use slides starting from the third one;
- do not place the slices in a water bath.

Conditions for transportation and storage of the initial

biological material:

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

ATTENTION! Avoid repeated freezing and thawing of samples.

FFPE-blocks transportation and storage conditions:

- at 15°C... 25°C — not more than 3 years.

8.2 Human DNA extraction from biological material

To isolate human genomic DNA from tissue samples fixed in 10% neutral formalin solution and embedded in a paraffin block — FFPE-block, it is recommended to use the following reagent kits:

- Reagent kit for human genomic DNA isolation from tissues fixed in formalin and embedded in paraffin (DNA-Tissue-F), manufactured by TestGene LLC, Russia (registration certificate No. RZN 2018/7772 dated 10.30.2018);
- Reagent kit for human genomic DNA isolation from tissues fixed in formalin and embedded in paraffin (DNA-Tissue-M), manufactured by TestGene LLC, Russia (registration certificate No. RZN 2021/14273 dated 06.05.2021).

It is necessary to strictly follow the protocol and the instructions of the used reagent kit during the DNA isolation procedure.

Conditions for DNA test samples possible storage

- at 2°C... 8°C — during 24 hours,
- at -18°C... -22°C — for 1 month,
- at -80°C — for a long time.

8.3 Interfering substances and restrictions on the tested material use

The potentially interfering substances effect on the Test-EGFR-tissue-multi reagent kit performance has been examined for potentially interfering substances that may originate from the following external and internal sources:

1) substances used in patient treatment (e.g., medicines);
2) substances found in specific sample types — in this case clinical sample contamination with hemoglobin can inhibit a PCR if not

sufficiently purified during the DNA isolation procedure;

3) substances added during sample preparation — paraffin, which is used for a FFPE block preparation.

Interfering substances concentrations are shown in the Table 6.

Table 7

Interfering substances	**Maximum concentration**
Endogenous interfering substances	
Hemoglobin	260 µl/ml
Exogenous interfering substances	
Substances added during sample preparation	
Paraffin	$1*10^{-4}$ µl/µl
Cancer treatment drugs	
Gefitinib (indicated for locally advanced or metastatic NSCLC with activating mutations presence in the *EGFR* gene)	0.05 mg/ml
Erlotinib (used for NSCLC, pancreatic cancer treatment)	0.02 mg/ml
Etoposide (used for ovarian cancer, small cell and non-small cell lung cancer, stomach cancer, etc. treatment)	0.02 mg/ml
Paclitaxel (used for ovarian cancer, breast cancer, NSCLC, cervical cancer, pancreatic cancer, Kaposi's sarcoma treatment)	0.006 mg/ml
Gemcitabine (used as therapy for pancreatic, bladder, NSCLC, ovarian, breast cancer)	0.04 mg/ml
Cisplatin (anticancer drug)	0.002 mg/ml

Based on the study results, potentially interfering substances found during the DNA isolation procedure from clinical material, evaluated at concentrations that are expected to occur during Test-EGFR-tissue-multi reagent kit normal use do not affect the test result.

Limitations on test material use:

- test material usage is not allowed under storage and transportation conditions violation (temperature, duration, repeated freezing and thawing);
- it is not allowed to use samples contaminated with extraneous biological material.
- tumor minimum concentration for analysis conduction — 20% based on the tumor material morphological examination results obtained by a histologist.

- test DNA purity ratio expressed in terms of optical densities (A260/280nm) and required for the test should be not less than 1.4;
- sufficient for the assay DNA concentration is 1-50 ng/μL;
- genomic DNA samples isolated from histologically confirmed tumor tissue should be used for analysis.

9. Kit components preparation for testing

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

ATTENTION! It is required to use only disposable sterile plastic consumables that have a special "DNase-free" label when working with DNA. It is mandatory to use a separate pipette tip with an aerosol barrier for each reaction component.

ATTENTION! Reaction mixture components should be mixed according to Table 6 right before test conduction.

Before preparing the reactions, PCR cabinet, equipment and materials contained in it should be wet cleaned using disinfectants suitable for use in PCR laboratories, and exposed to UV-radiation for 20-30 minutes.

1. Mix thoroughly the tubes contents with the isolated for test DNA, PCR buffer, primer-mixes, PC and NC, turn over each tube 10 times or mix using vortex at low speed for 3-5 seconds, then remove the drops from the test tube lids by short centrifugation.

2. Select the required number of 0.2ml PCR tubes for each multiplex according to the calculation: test samples number[7] + 1 PC + 1 NC.

Each sample is supplied with one or more multiplexes depending on the necessity to identify specific mutations (primer-mixes).

Table 8 shows PCR tubes layout for seven multiplexes.

Table 8 — The PCR tubes layout

Multiplex	Sample 1	Sample n	PC	NC
719 (detects G719S, G719C, G719D, G719A mutations status but does not differentiate them)	○	○	○	○
768 (S768I mutations status)	○	○	○	○
790 (T790M mutations status)	○	○	○	○
858 (L858R 2 mutations status, detects but does not differentiate them)	○	○	○	○
861 (L861Q mutation status)	○	○	○	○
Del (35 deletions status, detects but does not differentiate them)	○	○	○	○
Ins (4 insertions status, detects but does not differentiate them)	○	○	○	○

[7]To improve accuracy, it is recommended to analyze each sample in two repetitions.

10. **Testing procedure**

PCR testing includes following steps:

1. PCR Setup;
2. Real-Time DNA PCR amplification with hybridization-fluorescence detection of amplification products;
3. 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. Every reaction preparation requires:

1. PCR buffer — 4 µl,
2. Corresponding primer-mix (719, 768, 790, 858, 861, del, ins) — 10 µl,
3. Sample (PC, NC, DNA test sample) — 6 µl.

The reaction tubes should be prepared in the following order:

1. Label 0.2ml test tubes for PCR. For each multiplex select the required tubes number for test samples + 1 PC + 1 NC.

2. Add 4 µl of PCR buffer into each tube[8].

3. Add 10 µl of primer-mixes (719, 768, 790, 858, 861, del, ins) into the corresponding multiplexes tubes (Table 8).

4. Add 6 µl of isolated DNA into the corresponding test samples tubes[9]. Do not add DNA into the tubes for NC and PC.

5. Add 6 µl of PC into the corresponding test tubes of each multiplex used.

6. Add 6 µl of NC into the corresponding test tubes of each multiplex used.

7. Centrifugate the test tubes during 1-3 seconds to remove the drops from the walls. Use a microcentrifuge-vortex.

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[8] It is recommended to prepare a primer-mix and PCR-buffer mixture for each multiplex in a separate 1.5-2.0 ml tube according to the calculation: (n+3)x4 µl of PCR buffer + (n+3)x10 µl of the corresponding primer-mix, where n is the samples number. Mix using vortex. Remove drops by a short centrifugation. Add 14 µl in PCR tubes for a corresponding multiplex according to Table 7.

[9] To prevent PCR inhibition the sample volume can be reduced to 1-5 µl while the reaction amount is adjusted to 20 µl by DNase-free water deionized from a PC

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

(is performed in the PCR area — PCR amplification room)

1. Install tubes in the real-time PCR device reaction module. It is recommended to install the tubes in the center of the thermoblock to ensure that the tubes are pressed evenly by the heating lid.

2. Program the device to perform the corresponding fluorescence signal amplification and detection programs according to the used cycler instructions. PCR protocol is specified in Tables 9-10.

3. Specify the samples numbers and identifiers, mark the tubes location on the thermoblock matrix in accordance with their installation.

4. Make sure that the FAM/Green, HEX/Yellow detection channels are applied for the optical measurement parameter amplification program.

5. Start PCR with fluorescent signal detection.

6. At the end of the program, start analyzing the results.

Table 9 – PCR protocol for cyclers manufactured by "DNA-Technology"

Stage	**Temperature, ˚C**	**Time, min:sec**	**Detection channels**	**Total cycles number**
1	95	02:00	-	1
2	95	00:10	-	50
	64	00:15	FAM/Green, HEX/Yellow	

ATTENTION! Factory optical measurement exposure parameters for each channel should be used for devices manufactured by "DNA-Technology"

Table 10 — PCR protocol for other manufacturer's cyclers

Stage	**Temperature, ˚C**	**Time, min:sec**	**Detection channels**	**Total cycles number**
1	95	02:00	-	1
2	95	00:10	-	50
	62	00:15	FAM/Green, HEX/Yellow	

11. Result registration and interpretation

Results registration is carried out automatically upon PCR completion with the used device software.

Recommendations on setting the threshold line

For cyclers of any model, the threshold line is set individually for each detection channel at a level corresponding to 5-10% of the maximum fluorescence level obtained for the positive control sample in the last amplification cycle.

The results are interpreted using the FAM/Green and HEX/Yellow channels Ct values (Table 11).

First, the reaction passage and Ct values in control samples are evaluated. Test samples results interpretation starts only with the correct PC and NC passage.

ATTENTION! If Rotor-Gene Q and similar cyclers are used, activate the "Dynamic Tube" and "Noise slope correction" functions. Set 10% in the "Outlier Removal" section.

In case of using CFX 96 cycler you may need to align some graphs with incorrect slope using Baseline Threshold → Baseline Cycles settings.

Table 11 - Results interpretation in the FAM/Green and HEX/Yellow channels

Multiplex (primer-mix)	**A channel corresponding to a fluorophore**	
	FAM/ Green	**HEX/ Yellow**
719	G719S, G719C, G719D, G719A mutations in *EGFR* gene exon 18 (detects mutations but does not differentiate them)	SVC (human *EGFR* gene)
768	S768I mutation in the *EGFR* gene exon 20	SVC (human *EGFR* gene)
790	T790M mutation in the *EGFR* gene exon 20	SVC (human *EGFR* gene)
858	L858R mutations in the *EGFR* gene exon 21 (detects 2 mutations but does not differentiate them)	SVC (human *EGFR* gene)
861	L861Q mutation in the *EGFR* gene exon 21	SVC (human *EGFR* gene)
Del	Deletions in the *EGFR* gene exon 19 (detects 35 mutations but does not differentiate them)	SVC (human *EGFR* gene)
Ins	Insertions in the *EGFR*gene exon 20 (detects 4 mutations but does not differentiate	SVC (human *EGFR* gene)

	them)	

Results interpretation in control samples

The following results should be obtained for NC and PC (Table 12).

Table 12 — Test results for PC and NC

Control sample	Selected fluorophore	
	FAM/Green (G719S, G719C, G719C, G719D, S768I, T790M, L858R, L861Q mutations, del, ins)	HEX/Yellow (*EGFR* wild-type gene)
NC	Absent	Absent
PC	Ct ≤35	Ct ≤35

When obtaining values for the NC that differ from those mentioned in Table 12, the entire assay results are considered unreliable. In this case, special measures should be taken to eliminate possible contamination.

If PC values differ from those indicated in Table 12, repeated amplification of the entire sample batch is required. If after repeated amplification PC results differ from those indicated in Table 11, the reagents must be replaced.

Results interpretation

Result registration is carried out using the cycler software used for PCR conduction with real time detection.

The fluorescent accumulation curves are analyzed in two channels (Table 11):

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

- signal indicating DNA amplification products accumulation of the *EGFR* wild-type gene variants is registered in the HEX channel (sampling volume control — SVC)

The results are interpreted based on the presence or absence of the threshold line fluorescence curve intersection.

Reaction passage in the HEX/Yellow Ct≤35 (Ct ≤40 for 790, 861 multiplexes using CFX96, Rotor-Gene Q cyclers) indicates sufficient quality of the material intake, nucleic acid extraction efficiency and PCR inhibitors absence.

If there is no reaction in the HEX/Yellow channel or Ct>35 (Ct ≤40 for 790 multiplex using CFX96, Rotor-Gene Q cyclers) and at the same time there is no reaction in the FAM/Green channel, the result should be considered invalid, and a second test starting with DNA extraction should be conducted for the test sample.

Results interpretation principles are shown in Tables 13-19

Table 13 — Results interpretation principle for 719 multiplex (detects G719S, G719C, G719D, G719A mutations in the *EGFR*, gene exon 18 but does not differentiate them*)*

Ct Values		Result
Fluorescence channels **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct n ≤35	not considered	G719X mutation (G719S, G719C, G719D or G719A) in the *EGFR* gene is detected
Ct is absent	Ct ≤35	G719X mutation (G719S, G719C, G719D or G719A) in The *EGFR* gene is not detected or is below the detection limit
Ct in both specific channels is >35 or absent		Mutations presence result is invalid
Ct >35	Ct ≤35	G719X (G719S, G719C, G719D or G719A) mutation presence result in the *EGFR* gene is doubtful

Table 14 — 768 multiplex results interpretation principle (detects S768I mutations in the *EGFR* gene exon 20

Ct values		Result
Fluorescence channels **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct ≤35	not considered	S768I mutation in the *EGFR* gene is detected
Ct is absent	Ct ≤35	S768I mutation in the *EGFR* gene is not detected or is below the detection limit
Ct in both channels is >35 or absent		Mutation presence result is invalid
Ct >35	Ct ≤35	S768I mutationpresence result in the *EGFR* gene is doubtful

Table 15 —multiplex 790 results interpretation (detects T790M mutations in the *EGFR* gene exon 20)

Ct values		Result
Fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct ≤35	not considered	T790M mutation in the *EGFR* gene is detected
Ct is absent	Ct ≤35 – for DTprime, QuantStudio 5; Ct ≤40 – for CFX96, Rotor-Gene Q	T790M mutation in the *EGFR* gene is not detected or is below the detection limit
Ct >35	Ct >35 – for DTprime, QuantStudio 5; Ct >40 – for CFX96, Rotor-Gene Q	Mutation presence result is invalid
Ct >35	Ct ≤35 – for DTprime, QuantStudio 5; Ct ≤40 − for CFX96, Rotor-Gene Q	T790M mutations presence result in the *EGFR* gene is doubtful

Table 16 —multiplex 858 results interpretation (detects 2 L858R mutations in the *EGFR* gene exon 21 but does not differentiate them)

Ct Values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct ≤35	not considered	L858R mutation in the *EGFR* gene is detected
Ct is absent	Ct ≤35	L858R mutation in the *EGFR* gene is not detected or is below the detection limit
Ct in both channels is >35 or absent		Mutations presence result is invalid
Ct >35	Ct ≤35	L858R mutations presence result in the *EGFR* gene is doubtful

Table 17 —multiplex 861 results interpretation principle (L861Q in the *EGFR* gene exon 21)

Ct values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct ≤35	not considered	L861Q mutation in the *EGFR* gene is detected
Ct is absent	Ct ≤35 for DTprime, QuantStudio 5; Ct ≤40 – for CFX96, Rotor-Gene Q	L861Q mutation in the *EGFR* gene is not detected or is below the detection limit
Ct >35	not considered	L861Q mutations presence result in the *EGFR* gene is doubtful

Table 18 - Del multiplex results interpretation principle (detects 35 deletions in the *EGFR* gene exon 19 but does not differentiate them)

Ct Values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct < 40	not considered	Del mutation in the *EGFR* gene is detected
$(Ct^{FAM} - Ct^{HEX}) \leq 10$		
Ct is absent or $(Ct^{FAM} - Ct^{HEX}) > 10$	Ct ≤ 25	Del mutation in the *EGFR* gene is not detected or is below the detection limit
Ct is absent	25 < Ct ≤ 35	
$(Ct^{FAM} - Ct^{HEX}) > 10$	25 < Ct ≤ 35	Del mutations presence result in the *EGFR* gene is doubtful
Ct is absent or $(Ct^{FAM} - Ct^{HEX}) > 10$	Ct > 35 or absent	Mutations presence result is invalid

Table 19 - Ins multiplex results interpretation principle (detects 4 insertions in the *EGFR* gene exon 20 but does not differentiate them)

Ct Values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct ≤40	not considered	Ins mutation in the *EGFR* gene is detected
Ct is absent	Ct ≤35	Ins mutation in the *EGFR* gene is not detected or is below the detection limit
Ct >40 or absent	Ct>35 or absent	Mutations presence result is invalid
Ct >40	Ct ≤35	Ins mutations result in the *EGFR* gene is doubtful

It is recommended to repeat the isolated DNA PCR test to exclude false negative results. The reasons for obtaining an invalid result may be low DNA concentration, inhibitors' presence in a DNA sample obtained from clinical material; incorrect analysis protocol execution; non-compliance with the PCR temperature regime, etc.

If the result is invalid the conclusion is not issued. It is necessary to conduct the analysis again.

If an invalid result repeats, retest with another manufacturer's

reagent kit or using another method.

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

Obtained analysis result diagnostic value:

The obtained positive or negative test result can be used by a qualified specialist (oncologist), taking into account the clinical picture data and other research types to determine indications for targeted therapy with small molecule *EGFR* kinase inhibitors during examination of patients diagnosed with non-small cell lung cancer stage IB-IIIA and stage IV (Clinical recommendations: "Skin cancer and mucosal melanoma", C43, C51, C60.9, C63.2; "Thyroid cancer", C73; "Ovarian cancer/fallopian tube cancer/primary peritoneal cancer", C48, C56, C57; "rectal cancer", C20; "Prostate cancer", C61. Adults. 2021. Authors: All-Russian National Union "Association of oncologists of Russia", All-Russian Public Organization "Russian Society of Clinical Oncology" (approved by The Ministry of Health of the Russian Federation).

12. Storage, transportation and usage conditions

Storage

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

Store an opened kit under the same conditions as before opening.

Reagent kit stored under storage conditions violation cannot be used.

Transportation

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

Test-EGFR-tissue-multi reagent kit transportation is allowed at 2°C... 8°C during the entire shelf-life period. Transportation is allowed at 15°C... 25°C up to 5 days.

Atmospheric pressure is not under 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-EGFR-tissue-multi 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 an expired shelf life cannot be used.

Opened kit components shelf life

12 months from the acceptance date by the manufacturer's QCD if stored at 2°C... 8°C.

Ready for usage kit components shelf life

One 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-EGFR-tissue-multi 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-*EGFR*-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