Instruction for use

A reagent kit for determination of *BRAF* gene mutation status by PCR-RT in human genomic DNA from FFPE tissue samples

Test-*BRAF*-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
NC	positive control sample
PC	positive control sample
SVC	sampling volume control
ID	identification number
COSMIC	Catalog of Somatic Mutations in Cancer
FFPE-block	Formalin-Fixed Paraffin-Embedded tissue

Introduction

BRAF gene is a part of EGFR signaling pathway that regulates angiogenesis and cell proliferation. In a normal cell this signaling pathway is in a controlled-regulated state but when an activating mutation occurs in any region of this signaling pathway, the mutated gene starts to continuously produce permanently activated protein. This protein launches the excessive cell proliferation process that leads to tumors formation.

Mutations in the *BRAF* gene are present in about 50% of melanoma cases, 40% of papillary thyroid tumors, 30% of ovarian tumors, 10% of colorectal cancer and about 10% of prostate tumors.

Target analyte detected with the Test-*BRAF*-tissue-Multi-24 reagent kit is mutations in the *BRAF* gene — V600E, V600E complex (detects mutations V600E, V600E complex, but does not differentiate them), V600K, V600R, V600D, V600Dc (detects mutations V600R, V600D, V600Dc but does not differentiate them), V600M.

The target analyte **scientific validity** lies in its specificity (the DNA sequence uniqueness) in relation to the *BRAF* gene somatic mutations.

The *BRAF* gene (7q34) encodes a protein serine-threonine kinase, mutations in the activation domain that cause a stable cascade hyperactivation of mitogen-activated protein kinases MEK and ERK[1].

The *BRAF* gene belongs to proto–oncogenes — somatic activating mutations are found in this gene in about 7-8% of malignant tumors. They are mostly localized in the *BRAF gene* exon 15 (codons 600 and 601). The *BRAF* gene mutation status can be used as prognostic and especially as predictive biomarker — mutation presence is associated with potential resistance against any of receptor tyrosine kinase inhibitors, as well as against monotherapy with mTOR inhibitors[2].

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[1] Mazurenko N. N. "Genetic alterations and markers of melanoma// Advances in Molecular Oncology" - 2014. - vol 2 - p. 26-35.

[2] Kachko V.A., Zaretsky A.R., Vanushko V.E. Somatic mutation testing: the role in differential diagnosis of thyroid neoplasms// Endocrine Surgery — vol. 13 — №1 — p. 26-41

The most common mutations (over 97%) are localized in codon 600. About 90% of them are valine substitutions with glutamic acid — V600E. Less common nucleobase substitutions are — thymine V600K (about 8-20%), arginine V600R — 1%, leucine V600M — 0,3%, aspartic acid V600D — 0,1%[3].

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

Indications and contraindications for use

Intended use: Test-*BRAF*-tissue-Multi-24 reagent kit is designed for patients examination diagnosed with tumor diseases (melanoma, papillary thyroid cancer, ovarian, colorectal and prostate cancer) in order to determine an effective strategy for targeted therapy with *BRAF* and *MEK* kinase inhibitors and to predict treatment efficacy.

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

Population and demographic aspects of the reagent kit usage: no population or demographic usage aspects of the Test-*BRAF*-tissue-Multi-24 reagent kit were identified.

Sterility: the kit is not sterile.

[3] In G.K., Poorman K., Saul M. et al. Molecular profiling of melanoma brain metastases compared to primary cutaneous melanoma and to extracranial metastases // Oncotarget. – 2020. – Vol. 11. – № 33. – P. 3118–3128.

1. Intended use

Intended use: the Test-*BRAF*-tissue-Multi-24 reagent kit is designed for mutation status determination in the *BRAF* gene — V600E, V600E complex (detects V600E, V600E complex mutations, but does not differentiate them), V600K, V600R, V600D, V600Dc (detects V600R, V600D, V600Dc mutations, but does not differentiate them), V600M by real-time multiplex polymerase chain reaction with hybridization fluorescence detection in the genomic DNA sample isolated from tissue fixed in 10% formalin solution and embedded in a paraffin block (FFPE-block) when examining patients with tumor diseases (melanoma, papillary thyroid cancer, ovarian cancer, colorectal cancer, prostate cancer) in order to determine an effective strategy for targeted therapy by *BRAF* and *MEK* kinase inhibitors and to predict the treatment effectiveness (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).

Functional use: obtained results can be used for patients diagnosed with tumor diseases (melanoma, papillary thyroid cancer, ovarian cancer, colorectal and prostate cancer) examination in order to determine an effective strategy for targeted therapy by *BRAF* and *MEK* kinase inhibitors to predict the treatment effectiveness.

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

Status determination of the *BRAF* gene mutations — V600E, V600E complex (detects V600E, V600E complex mutations, but does not differentiate them), V600K, V600R, V600D, V600Dc (detects V600R, V600D, V600Dc mutations, but does not differentiate them), V600M by RT-PCR with hybridization-fluorescence detection in a genomic DNA probe isolated from biomaterial includes three stages:

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

DNA samples are used for conducting genomic DNA regions 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 a PCR-optimized buffer. The uracil-DNA glycosylase enzyme presence prevents obtaining false positive results during amplification products contamination. The enzyme is completely inactivated during the first cycle of DNA denaturation and does not interfere with the current reaction products amplification.

The primer mix contains fluorescent labeled oligonucleotide probes that hybridize with a complementary region of the amplified target DNA and get 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 V600E, V600E complex (detects V600E, V600E complex mutations, but does not differentiate them), V600K, V600R, V600D, V600Dc (detects V600R, V600D, V600Dc mutations, but does not differentiate them), V600M as well as a the *BRAF* wild-type gene used as a sampling 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**
600E/Ec	V600E, V600Ec mutations in the *BRAF* gene (detects mutations but does not differentiate them)	SVC (human *BRAF* gene)
600K	V600K mutation in the *BRAF* gene	SVC (human *BRAF* gene)
600R/D/Dc	V600R, V600D, V600Dc mutations in the *BRAF* gene (detects mutations but does not differentiate them)	SVC (human *BRAF* gene)
600M	V600M mutation in the *BRAF* gene	SVC (human *BRAF* gene)

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

Method limitations

Mutation detection depends on the sample integrity and on the amplified DNA amount in a sample. The required for the assay isolated DNA purity is 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 other region results of the same tumor. Also, tumor samples may contain normal (non-tumor) tissue. When using a genomic DNA sample isolated from tissue that does not contain tumor, Test-*BRAF*-tissue-Multi-24 reagent kit will not be able to detect mutations in the *BRAF* 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-*BRAF*-tissue-Multi-24 reagent kit cannot be used for any pathology diagnostics. The reagent kit is designed only for the *BRAF* gene V600E, V600E complex (detects mutations V600E, V600E complex, but does not differentiate them), V600K, V600R, V600D, V600Dc (detects V600R, V600D, V600Dc mutations but does not differentiate them), V600M mutation status qualitative detection. Package integrity violation during transportation.

The reagent kit is not allowed to use after the expiration date or under the storage conditions violation.

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

Test-*BRAF*-tissue-Multi-24 reagent kit comes in 1 configuration.

Test samples number

The reagent kit is designed for 28 reactions of each multiplex sample (600E/Ec — V600E, V600Ec mutations (detects V600E, V600E mutations but does not differentiate them); 600K — V600K mutation; 600R/D/Dc — V600R, V600D, V600Dc mutations (detects V600R, V600D, V600Dc mutations but does not differentiate them); 600M — V600M mutation) that corresponds to 24 test samples with NC and PC in each run or 9 single runs with NC and PC.

Reagent kit components

Table 2 – Test-*BRAF*-tissue-Multi-24 reagent kit components

No.	Reagent	Description	Quantity, Volume
1	PCR buffer	Transparent colorless liquid	1 tube, 450 µl
2	600E/Ec Primer-mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl
3	600K Primer Mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl
4	600R/D/Dc Primer mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl
5	600M Primer Mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl
6	PC	Transparent colorless liquid	1 tube, 220 µl
7	NC	Transparent colorless liquid	1 tube, 220 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 a thermostable hot-start DNA polymerase, deoxynucleotide triphosphates, Uracil-DNA glycosylase and an optimized buffer.

600E/Ec primer-mix is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the *BRAF* gene V600E, V600Ec mutations detection (detects

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

600K primer-mix is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the *BRAF* gene V600K mutations detection (detection is carried out in the FAM/Green channel) and for the *BRAF* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

600R/D/Dc primer-mix is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the *BRAF* gene V600R, V600D, V600Dc mutations (detects mutations but does not differentiate them) (detection is carried out in the FAM/Green channel) and for the *BRAF* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

600M primer-mix is ready to use and contains multiplex primers and probes mixture — primers and fluorescent-labeled oligonucleotide probes for the *BRAF* gene V600M mutation (detection is carried out in the FAM/Green channel) and for the *BRAF* wild-type gene used as sampling volume control (SVC) (detection is carried out in the HEX/Yellow channel).

Reaction passage in the HEX/Yellow Ct≤35 indicates sufficient material collection quality, nucleic acid extraction efficiency and PCR inhibitors absence.

If there is no reaction in the HEX/Yellow channel or Ct>35 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.

Positive control sample (PC) is ready to use and is a plasmid DNA mixture with synthetic amplified DNA fragment inserts: a human *BRAF*

wild-type gene region, specific human *BRAF* gene regions with V600E, V600Ec, V600K, V600R, V600D, V600Dc, V600M mutations enclosed in the pAl-TA plasmid vector. Contains 12.5% of mutated and 87.5% of wild-type DNA 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-*BRAF*-tissue-Multi-24 reagent kit components

Indicator name	**Characteristics and standards**		**Clause in Technical Specification (TS)**
1.1 Technical characteristics			
Reagent	**Appearance**	**Quality, volume, µl (±5%)**	
PCR buffer	Transparent colorless liquid	1 tube, 450 µl	Section 7, clause 7.6
600E/Ec Primer-mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl	Section 7, clause 7.6
600K Primer-mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl	Section 7, clause 7.6
600R/D/Dc Primer-mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl	Section 7, clause 7.6
600M Primer-mix	Transparent colorless liquid, may have a lilac shade	1 tube, 280 µl	Section 7, clause 7.6
PC	Transparent colorless liquid	1 tube, 220 µl	Section 7, clause 7.6

NC	Transparent colorless liquid	1 tube, 220 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). In the HEX/Yellow channel Ct ≤35.		Section 7, clause 7.8.2

NOTE: a 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 deviation 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 concentration of U-937 stock solution (manufactured by SibEnzyme, Russia) used as DNA copies with the *BRAF* wild-type gene, and plasmid concentrations with the *BRAF* mutated gene sequence insertion: V600E, V600Ec, V600K, V600R, V600D, V600Dc, V600M, that are used as DNA copies with the *BRAF* gene mutations in PC.

Subsequent PCR-RT multiplex allele-specific polymerase chain reaction conduction confirmed that a positive control sample (PC) ensures the Test-*BRAF*-tissue-Multi-24 reagent kit stable work and is a plasmid DNA mixture with synthetic inserts of the amplified DNA fragments: human *BRAF* wild-type gene, specific fragments with human *BRAF* gene mutations: V600E, V600Ec, V600K, V600R, V600D, V600Dc, V600M, enclosed in the pAl-TA plasmid vector.

4.2 Analytical efficiency characteristics

4.2.1 Analytical specificity

Specific to V600E, V600E complex (detects V600E, V600E complex mutations but does not differentiate them), V600K, V600R, V600D, V600Dc (detects V600R, V600D, V600Dc mutations but does not differentiate them), V600M and to a human *BRAF* wild-type gene.

The *BRAF* 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 the Table 4.

Table 4 — a list of differentiated mutations with ID* indication

A list of mutations differentiated using the Test-*BRAF*-tissue-Multi-24 reagent kit			COSMIC ID*
Localizaion	Amino acid substitution	Nucleotide substitution	
Exon 15	p.V600E	c.1799T>A	476
	p.V600E (Ec)	c.1799_1800TG>AA (complex)	475
	p.V600R	c.1798_1799GT>AG	474
	p.V600D	c.1799_1800TG>AT	477
	p.V600D (Dc)	c.1799_1800TG>AC (complex)	308550
	p.V600K	c.1798_1799GT>AA	473
	p.V600M	c.1798G>A	1130

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

4.2.2 Analytical sensitivity

10 copies of the BRAF 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).

While conducting accuracy testing under reproducibility conditions complete intra-stage, inter-stage and inter-series reproducibility was observed, 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 lowest frequency of declared alleles in a sample in the *BRAF* genes, which the reagent kit is able to detect — 5%.

4.2.7 The product-specificity in relation to skin microflora has been confirmed by DNA strains:

- from the "GKPM-Obolensk" collection: *Staphylococcus aureus* Wood 46 ATCC 10832 (strain number B-4442), *Staphylococcus epidermidis* ATCC 14990 (strain number B-4510),
- from the "Thermo Fisher Scientific" collection (USA): *Streptococcus pyogenes* ATCC 19615 PK/5 (lot: 251028),
- from the "Microbiologics" collection (USA): *Streptococcus agalactiae* Group B ATCC 13813 (№ 0370),

in the maximum pathogen concentration of 10^6 cells per ml.

The specificity of the product against colon and rectal microflora was confirmed by DNA strain testing:

– from the "GKPM-Obolensk" collection: *Escherichia coli* M-17 (strain number B-2929), *Shigella sonnei* «S-form» (strain number B- 4385), Salmonella enterica subsp. enterica serovar Typhimurium 79 (strain number B-4376), *Shigella flexneri* 1a 8516 (strain number B-4388), *Candida albicans* NCTC 885-653 (strain number B-7940),

– from the National bioresource center "All-Russian Collection of Industrial Microorganisms (BRC VKPM)": Enterococcus faecium (Streptococcus faecium) (strain number B-4954), Lactococcus lactis subsp. lactis (strain number B-8837), *Salmonella typhimurium* TA102 (strain number B-5393).

Non-specific reactions were not detected in concentration from 10^6 to 10^7 cells per 1 ml.

The above-mentioned microorganisms do not affect the Test-*BRAF*-tissue-multi-24 reagent kit ability to differentiate the *BRAF* gene mutated and wild-type variants.

4.2.9. The kit was tested using the *BRAF* gene homologous proteins. Test-*BRAF*-tissue-multi-24 reagent kit does not cross-react with homologous *BRAF* genes – *ARAF, RAF1.*

4.2.10 interfering substances effect

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

4.3. Clinical Effectiveness

62 tissue samples of patients with tumor diseases (melanoma, papillary thyroid cancer, ovarian cancer, colorectal cancer, prostate cancer) fixed in 10% formalin solution and embedded in a paraffin block (FFPE-block) were used for clinical essay conduction.

To assess inter-lot repeatability clinical samples studies were conducted with the reagent kit in two series.

The quality, safety and efficiency control were carried out in 124 tests.

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%.

Table 5 - Clinical efficiency

Mutation	**Positive samples observation number**	**Negative samples observations number**	**Diagnostic sensitivity** with 95% confidence probability	**Diagnostic specificity** with 95% confidence probability
V600E (c.1799T>A)	70	60	100% (95% diagnostic interval:94,87%-100%)	100% (95% diagnostic interval: 94,04%-100%)
V600E complex (c.1799_1800TG>AA)	26	104	100% (95% diagnostic interval: 86,77%-100%)	100% (95% diagnostic interval: 96,52%-100%)

V600R (c.1798_1799delinsAG)	4	126	100% (95% diagnostic interval: 39,76%-100%)	100% (95% diagnostic interval: 97,11%-100%)
V600D (c.1799_1800delinsAT)	2	128	100% (95% diagnostic interval: 97,11%-100%)	100% (95% diagnostic interval: 97,16%-100%)
V600Dc (c.1799_1800delinsAC)	6	124	100% (95% diagnostic interval: 54,07%-100%)	100% (95% diagnostic interval: 97,07%-100%)
V600K (c.1798_1799GT>AA)	14	116	100% (95% diagnostic interval: 76,84%-100%)	100% (95% diagnostic interval: 96,87%-100%)
V600M (c.1798G>A)	2	128	100% (95% diagnostic interval: 97,11%-100%)	100% (95% diagnostic interval: 97,16%-100%)

5. Risks associated with the reagent kit use

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-*BRAF*-tissue-multi-24 reagent kit for *BRAF* gene mutation 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 the Test-*BRAF*-tissue-multi-24 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-*BRAF*-tissue-multi-24 reagent kit have low vapor pressure and exclude the possibility of inhalation poisoning.

The reagents included in the Test-*BRAF*-tissue-multi-24 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:

1. 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.

2. use the kit strictly for its intended purpose, according to this instruction;

3. do not use the kit if the package is violated;

4. 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);

5. do not use the kit after the expiration date;

6. 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

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[4] with real-time fluorescence detection in the channels corresponding to the FAM/Green and HEX/Yellow fluorophores:

- DTprime cycler (DNA-Technology LLC, Russian Federation, registration certificate № FSR 2011/10229 dated 03.03.2011);
- CFX 96 cycler (BioRad, USA, registration certificate № FSZ 2008/03399 dated 21.06.2016);
- Rotor-Gene Q cycler (Qiagen, Germany, registration certificate № FSZ 2010/07595 dated 10.08.2010);
- QuantStudio 5 cycler (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.

1. Disposable tips with an aerosol barrier up to 1,000 µl, 200 µl, 20 µl and 10 µl (e.g., Axygen, USA);
2. 1.5ml Disposable Eppendorf type sterile tubes;
3. 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),

[4] 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.

- 0.2ml PCR strip tubes;

4. Lab coat and disposable talc-free gloves;
5. Container with disinfectant;
6. 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).

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 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.

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

Conditions for test DNA samples possible storage

- at 2°C... 8°C — during 24 hours,
- at -20°C — for a long time.

8.3. Interfering substances and restrictions on the tested material use

The potentially interfering substances effect on the Test-*BRAF*-tissue-multi-24 reagent kit performance has been examined for potentially interfering substances that may originate from the following external and internal sources:

1) substances used in a patient treatment (e.g., medicines);

2) substances found in specific sample types — in this case a 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.

Examined interfering substances concentrations are shown in the Table 6.

Table 6

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	
Zelboraf (indicated for inoperable or metastatic melanoma)	0.048 mg/ml
Tafinlar (used for the unresectable or metastatic melanoma treatment, advanced non-small cell lung cancer)	0.01 mg/ml
Kotellik (used as therapy for inoperable or metastatic melanoma with the BRAF gene V600 mutation)	0.004 mg/ml
Tecentric (used for melanoma, hepatocellular carcinoma, breast cancer treatment, etc,.)	0.168 mg/ml
Mekinist (indicated for patients with unresectable or metastatic melanoma with the BRAF gene V600 mutation treatment)	0.0001 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-*BRAF*-tissue-multi-24 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;
- use genomic DNA test samples isolated from histologically confirmed tumor tissue;
- tumor minimum content for assay conduction — 20% based on the tumor material morphological examination results obtained by a histologist.

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 7 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 according to the calculation for each multiplex: test samples number[5]+ 1 PC + 1 NC.

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

Table 7 shows PCR tubes layout for six multiplexes.

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

Table 7 - The PCR tubes layout

Multiplex	Sample 1	Sample n	PC	NC
600E/Ec (V600E, V600Ec mutations status)	○	○	○	○
600K (V600K mutation status)	○	○	○	○
600R/D/Dc (V600R, V600D, V600Dc mutations status)	○	○	○	○
600M (V600M mutation status)	○	○	○	○

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

(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 (600E/Ec, 600K, 600R/D/Dc, 600M) — 10 µl,
3. Sample (PC, NC, DNA test sample) — 6 µl.

The reaction tubes should be prepared according to Table 7 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[6].

3. Add 10 µl of primer-mixes (600E/Ec, 600K, 600R/D/Dc, 600M) into the corresponding multiplexes tubes.

4. Add 6 µl of isolated DNA[7] into the corresponding test samples tubes. 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. Add 6 µl of SC into corresponding test tubes of each multiplex used.

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

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

[6] 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.

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

2. Program the device to perform the corresponding PCR program according to the instructions for the used cycler. PCR protocol is specified in Tables 8-9.

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 a fluorescent signal detection.

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

Table 8 – 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	00:05	-	50
	64	00:15	FAM/Green, HEX/Yellow	

ATTENTION! For devices manufactured by DNA-Technology factory optical measurement exposure parameters for each channel should be used.

Table 9 — PCR protocol for other manufacturers cyclers

Stage	**Temperature, ˚C**	**Time, min:sec**	**Detection channels**	**Total cycles number**
1	95	02:00	-	1
2	95	00:05	-	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 models, the threshold line is set individually for each detection channel at a level of curves transition to exponential growth.

The results are interpreted using the FAM/Green and HEX/Yellow channels Ct values (Table 10).

First, the reaction rate 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.

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

Table 10 - Results interpretation in the FAM/Green and HEX/Yellow channels

Multiplex (primer-mix)	**A channel corresponding to a fluorophore**	
	FAM/ Green	**HEX/ Yellow**
600E/Ec	V600E, V600Ec mutations in the *BRAF* gene (identifies mutations, but does not differentiate them)	SVC (human *BRAF* gene)
600K	V600K mutation in the *BRAF* gene	SVC (human *BRAF* gene)
600R/D/Dc	V600R, V600D, V600Dc mutations in the *BRAF* gene (identifies mutations, but does not differentiate them)	SVC (human *BRAF* gene)
600M	V600M mutation in the *BRAF* gene	SVC (human *BRAF* gene)

Results interpretation in control samples

The following results should be obtained for NC and PC (Table 11).

Table 11 — Test results for PC and NC

Control sample	Selected fluorophore	
	FAM/Green (V600E, V600Ec, V600K, V600R, V600D, V600Dc, V600M mutations)	HEX/Yellow (*BRAF* wild-type gene)
NC	Absent	Ct >35 or absent
PC	Ct ≤35	Ct ≤35

When obtaining NC values that differ from those mentioned in Table 11, 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 11, 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-RT conduction.

The fluorescent accumulation curves are analyzed in two channels (Tables 12-16):

- A signal indicating DNA amplification products accumulation of mutated *BRAF* gene variants is registered in the FAM channel.
- A signal indicating DNA amplification products accumulation of the *BRAF* wild-type gene is registered in the HEX channel (used as sampling volume control — SVC)

The results interpretation is based on the presence or absence of the fluorescence curve intersection of the threshold line.

Reaction passage in the HEX/Yellow Ct≤35 indicates the material intake sufficient quality, the nucleic acid extraction efficiency and the PCR inhibitors absence.

If there is no reaction in the HEX/Yellow channel or Ct>35 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 methods are shown in Tables 12-16.

Table 12 — 600E/Ec multiplex results interpretation principle (detects V600E, V600Ec mutations in the *BRAF* gene but does not differentiate them*)*

Ct Values		Result
Fluorescence channels **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct FAM/Green ≤35	not considered	V600E, V600Ec mutations in the *BRAF* gene are detected
Ct FAM/Green is absent	Ct ≤35	V600E, V600Ec mutations in the *BRAF* gene are not detected or are below the detection limit
Ct in both channels is >35 or absent		Mutations presence result is invalid
Ct FAM/Green >35	Ct ≤35	V600E, V600Ec mutations presence in the *BRAF* gene is doubtful

Table 13 — 600E/Ec multiplex results interpretation principle (detects V600K mutation in the *BRAF* gene)

Ct Values		Result
Fluorescence channels **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct FAM/Green ≤35	not considered	V600K mutation in the *BRAF* gene is detected
Ct FAM/Green is absent	Ct ≤35	V600K mutation in the *BRAF* gene is not detected or is below the detection limit
Ct in both channels is >35 or absent		Mutations presence result is invalid

Ct FAM/Green >35	Ct ≤35	V600K mutation presence in the *BRAF* gene is doubtful

Table 14 - 600R/D/Dc multiplex results interpretation principle (detects V600R, V600D, V600Dc mutations in the *BRAF* gene but does not differentiate them)

Ct Values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct FAM/Green ≤35	not considered	V600R, V600D, V600Dc mutations in the *BRAF* gene are detected
Ct FAM/Green is absent	Ct ≤35	V600R, V600D, V600Dc mutations in the *BRAF* gene are not detected or are below the detection limit
Ct in both channels is >35 or absent		Mutations presence result is invalid
Ct FAM/Green >35	Ct ≤35	V600R, V600D, V600Dc mutations presence in the *BRAF* gene is doubtful

Table 15 — 600M multiplex results interpretation (detects V600M mutation in the *BRAF* gene)

Ct Values		Result
fluorescence channel **(FAM/Green)**	SVC channel **(HEX/Yellow)**	
Ct FAM/Green ≤35	not considered	V600M mutation in the *BRAF* gene is detected
Ct FAM/Green is absent	Ct ≤35	V600M mutation in the *BRAF* gene is not detected or is below the detection limit
Ct in both channels is >35 or absent		Mutation presence result is invalid
Ct FAM/Green >35	Ct ≤35	V600M mutations presence result in the *BRAF* gene is doubtful

It is recommended to repeat PCR test of the isolated DNA preparation to exclude false negative results.

The reasons for obtaining an invalid result may be low DNA concentration, inhibitors' presence in the 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.

Obtaining a positive result in the FAM channel via several primer-mixes (multiplexes) in one sample within one run is possible for some devices if mutation concentration in the sample is high. There may be a slight cross-specificity in reaction mixtures detecting nucleotide substitution in the same DNA sequence position within a single codon. In that case non-specific rises using other primer mixes (multiplexes) are not taken into account while interpreting the result and only the main mutation is shown, i.e. result is valid for the mixture with the lowest Ct index in the FAM channel. An example of cross results for the samples with high concentration of one of the detected mutations is shown in Appendix A.

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

Obtained analysis result diagnostic value:

Obtained positive or negative test result can be used by a qualified specialist (oncologist), taking into account the data of the clinical picture and other types of research to determine an effective strategy for targeted therapy by *BRAF* and *MEK* kinase activity inhibitors and to predict the treatment effectiveness during examination of patients diagnosed with tumor diseases (melanoma, papillary thyroid cancer, cancer ovarian cancer, colorectal cancer, prostate cancer) (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-*BRA*F-tissue-multi-24 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 same conditions as before opening.

Reagent kit stored under storage conditions violation cannot be used.

Transportation

Test-*BRA*F-tissue-multi-24 reagent kit can be transported by all types of covered vehicles in accordance with the transportation rules applicable for the vehicle type.

Test-*BRA*F-tissue-multi-24 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-*BRAF*-tissue-multi-24 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-*BRAF*-tissue-multi-24 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

https://testgene.com/

Technical Support Service:

Phone number: +7 927 981 58 81

E-mail: help@testgen.ru

Annex A

Table 1. An example of a cross-specific signal with a high concentration of the BRAF gene individual mutations in the sample (data for a CFX 96 cycler (Bio-Rad)).

Reaction mixture (primer-mix)	**NC**	**Mutation-rich sample**													
		V600E mutation		**V600Ec mutation**		**V600K mutation**		**V600D mutation**		**V600Dc mutation**		**V600R mutation**		**V600M mutation**	
	FAM/ HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)	FAM (Ct)	HEX (Ct)
600 E/Ec	-	24.1	24.2	23.3	24.4	-	24.5	33.6	24.3	33.1	24.7	-	24.6	-	24.5
600 K	-	33.6	24.4	-	24.6	25	24.6	-	24.5	-	24.9	27.1	24.8	26,3	24.5
600 D, Dc, R	-	-	24.4	34.8	24.8	-	24.6	26	24.5	25.1	24.7	23.2	24.8	-	24.5
600 M	-	-	24.5	-	24.5	33.8	24.6	-	24.3	-	24.9	33.6	24.9	22.9	24.8