APPROVED BY
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INSTRUCTIONS FOR USE

Reagent kit for Chikungunya, Zika, Dengue virus RNA detection by RT-PCR-RT

Tropic-test

Version 1 dated 11.05.2024

1) Name and (or) trade name of an in vitro medical device

Reagent kit for Chikugunya, Zika, and Dengue virus detection by RT-PCR-RT Tropic-test

Short name — Tropic-test

2) Information on the reagent kit manufacturer

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/

3) Intended use

Intended use: reagent kit for qualitative detection of Chikungunya, Zika and Dengue virus RNA (1-4 types) specific regions by one-step reverse transcription method — real-time multiplex polymerase chain reaction with hybridization-fluorescence detection (RT-PCR-RT) in a sample of nucleic acids isolated from human blood plasma in patients suspected with infection caused by Chikungunya, Zika and Dengue viruses.

Functional use: obtained results can be used for an infection diagnostics caused by Chikungunya, Zika and Dengue viruses.

Target analyte: Chikungunya, Zika and Dengue virus RNA.

Chikungunya fever is a mosquito–borne viral disease caused by RNA-virus that belongs to the Togaviridae family (*Alphavirus* genus).

Zika fever is a mosquito–borne viral disease caused by RNA-virus that belongs to the Flaviviridae family (Flavivirus genus).

Dengue fever is a mosquito-borne disease caused by RNA-virus that belongs to the Flaviviridae family (Flavivirus genus).

Specific disorder, condition, or risk factor detection, determination, or differentiation the in vitro diagnostic medical device is designed for —Tropic-test reagent kit is designed for RNA qualitative detection by one-step reverse transcription — multiplex polymerase chain reaction with real-time hybridization-fluorescence detection (RT-PCR-RT) in order to diagnose Chikungunya fever caused by the Chikungunya virus (CHIKV), Zika fever caused by the Zika virus (ZIKV), Dengue fever caused by Dengue virus (DENV).

Is the device designed for qualitative, semi–quantitative or quantitative detections purpose of use — Tropic-test reagent kit is designed for RNA qualitative detection.

Test sample type — material for RT-PCR is RNA samples isolated from blood plasma (for configuration form 1); blood plasma samples (for configuration form 2).

Indications for use — Tropic-test reagent kit is recommended for use in patients with viral disease clinical symptoms, suspected of infection caused by Chikungunya, Zika and Dengue viruses.

Contraindications for use: none were identified.

4) Tropic-test reagent kit is designed for in vitro diagnostics in clinical laboratories.

5) Reagent kit potential consumers:

The kit is intended for professional use in medical centers and clinical diagnostic laboratories. The professional level of potential consumers is a clinical laboratory diagnostics doctor, a medical laboratory technician.

6) Method principle

Method

One-step reverse transcription — multiplex polymerase chain reaction in real time with hybridization-fluorescence detection (RT-PCR-RT).

Detection principle

Chikungunya, Zika and Dengue virus RNA detection is carried out by a one-step reverse transcription reaction followed by a real-time polymerase chain reaction in a single tube.

RT-PCR-buffer 5x contains all the main reagents including warm-start revertase, thermostable hot-start DNA polymerase, deoxynucleotide triphosphates and an optimized buffer (in the configuration form 2 deoxynucleotide triphosphates mixture is included in Primer-mix).

The Primer-mix contains fluorescent labeled oligonucleotide probes that hybridize with a complementary region of an amplified target DNA and get destroyed by *Taq*-polymerase. The dye and quencher separate, and fluorescence intensity increases. It allows to register specific amplification product accumulation by measuring the fluorescent

signal intensity in real time.

The kit contains reagents for qualitative detection of Chikungunya, Zika, and Dengue virus RNA and an internal control sample (ICS) RNA fragment: Chikungunya virus amplification products are recorded in the channel corresponding to the Cy5/Red fluorophore, Zika virus amplification products are recorded in the channel corresponding to the ROX/Orange fluorophore, Dengue virus amplification products are recorded in the channel corresponding to the FAM/Green fluorophore, ICS amplification products are recorded in the HEX/Yellow detection channel (Table 1).

Table 1 – Studied targets

A channel corresponding to a fluorophore			
FAM / Green	ROX / Orange	Cy5 / Red	HEX / Yellow
Dengue virus RNA	Zika virus RNA	Chikungunya virus RNA	ICS

ICS allows to assess the inhibitors presence in the sample that can lead to false negative results and/or the RNA isolation effectiveness.

RT-PCR reaction time ranges from 80 to 100 minutes, depending on the used cycler model (excluding sample preparation).

7) Reagents, calibrators, and control materials description

Configuration forms

The reagent kit is designed in 2 configuration forms:

1) Configuration form 1

Tropic-test-Classic reagent kit configuration form components.

2) Configuration form 2 - does not require RNA isolation from biological material (direct RT-PCR-RT)

Tropic-test-Cito reagent kit configuration form components.

Tests number

Each Tropic-test reagent kit configuration form (Tables 2-3) are designed to perform 96 reactions, it equates to detection of 94 test samples, negative and positive control samples during a single cycler run for 96 wells or 32 single test sample detections with negative and positive control samples in each run.

Reagent kit components

Table 2 – Tropic-test-Classic reagent kit (configuration form 1)

No.	Reagent	Description	Quantity, volume
Tropic-test-Classic RT-PCR reagent kit components			
1.	RT-PCR-buffer 5x	Transparent colorless liquid	1 tube, 480 µl
2.	Primer-mix	Transparent liquid, may have a lilac shade	1 tube, 480 µl
3.	PC	Transparent colorless liquid	1 tube, 480 µl
4.	NC	Transparent colorless liquid	2 tubes, 1600 µl
5.	ICS	Transparent colorless liquid	1 tube, 940 µl

Table 3 – Tropic-test-Cito reagent kit (configuration form 2)

No.	Reagent	Description	Quantity, volume
Tropic-test-Cito RT-PCR reagent kit components			
1.	RT-PCR-buffer-Cito 5x	Transparent colorless liquid	1 tube, 480 µl
2.	Primer-mix	Transparent liquid, may have a lilac shade	1 tube, 480 µl
3.	PC	Transparent colorless liquid	1 tube, 480 µl
4.	NC	Transparent colorless liquid	1 tube, 480 µl
5.	ICS	Transparent colorless liquid	1 tube, 940 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.

RT-PCR-buffer 5x is ready for use and contains all the necessary reagents including warm-start revertase, thermostable hot-start DNA polymerase, deoxynucleotide triphosphates and an optimized buffer.

RT-PCR-buffer-Cito 5x is ready for use and contains a genetically modified MMLV revertase with suppressed RNase H activity, a hot-start DNA polymerase and a buffer that allows to conduct RT-PCR in a large number of inhibitors presence.

Primer-mix is ready for use and contains primers and probes designed to identify specific targets:

1. Primers and a probe for a specific region fragment of Chikungunya virus genomic RNA. Detection is carried out in the Cy5/Red channel.
2. Primers and a probe for a specific region fragment of Zika virus genomic RNA. Detection is carried out in the ROX/Orange channel.
3. Primers and a probe for a specific region fragment of Dengue virus genomic RNA. Detection is carried out in the FAM/Green channel.
4. Primers and a probe for ICS. Detection is carried out in the HEX/Yellow channel.

Primer-mix for configuration form 2 additionally contains deoxynucleotide triphosphates.

Positive control sample (PC) is ready for use and contains Chikungunya, Zika, and Dengue virus genome specific fragments and ICS specific fragments detected by a reagent kit. PC contains in a TE-buffer (10 mM Tris, 1 mM EDTA) with addition of 0.05% sodium azide.

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

Internal control sample (ICS) is a ready for use armored RNA.

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

ATTENTION! Use reagents from the same reagent kit configuration form to conduct a reaction.

8) Materials and special materials required for testing (assay), but not included in the in vitro diagnostic medical device delivery set

RT-PCR-RT equipment:

1. Class II and III biological safety PCR cabinet (e.g. BMB-II-"Laminar-C"-1,2, Lamsystems, Russia).
2. Vortex (e.g. TETA-2, Biocom, Russia).
3. A set of electronic or automatic variable volume dispensers (e.g. Eppendorf, Germany).
4. Refrigerator for +2°C...+8°C with a freezer for below -16°C.
5. Cycler[1] with real-time fluorescence detection in the channels corresponding to FAM/Green, ROX/Orange, Cy5/Red, HEX/Yellow fluorophores, e.g., CFX96 (BioRad, USA), DTprime cycler (DNA-Technology LLC, Russia), Rotor-Gene Q cycler (Qiagen, Germany), QuantStudio 5 (Thermo Scientific, USA).

Materials and reagents not included in the kit:

ATTENTION! It is required to use only disposable sterile plastic consumables that have a special "Dnase-" and "RNase-free" label when working with RNA.

1. Disposable tips with an aerosol barrier up to 1 000 µl, 200 µl, 20 µl and 10 µl (e.g., Axygen, USA);
2. 1.5 or 2.0 ml disposable Eppendorf type sterile tubes;
3. Thin-walled PCR plates with an optically transparent lid (if detecting through a lid) or with optically transparent walls (if detecting through a tube wall): 0.1 or 0.2 ml PCR tubes or 0.1 or 0.2ml PCR tube strips or PCR plates with an optically transparent film (e.g. Axygen, USA), compatible with the used cycler;
4. Lab coat and disposable talc-free gloves;
5. Container with disinfectant;
6. Test tube rack for 0.1 or 0.2 ml tubes or for 0.1 or 0.2 ml tube strips (e.g., InterLabService, Russia);
7. Test tube rack for 1.5 ml tubes and tips;
8. Magnetic Eppendorf type 1.5 - 2.0 ml tube rack;

[1] 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 test performance.

9. Reagent kit for RNA extraction for configuration form 1.

9) Information for in vitro diagnostic medical device identification in order to obtain a safe combination and/or information about known limitations on combinations

9.1 To extract RNA it is recommended to use reagent kits for RNA isolation from blood plasma (e.g., NA-Extra reagent kit for DNA/RNA isolation from clinical material according to TS 21.20.23-013-97638376-2019, manufactured by TestGene LLC, Russia (registration certificate No. RZN 2021/15428 dated September 24, 2021):

- RNA isolation purity expressed in terms of optical densities (A260/280nm) is at least 1.6
- RNA isolation efficiency is at least 20%.

9.2 It is required to use only disposable sterile plastic consumables that have a special "RNase-free" label when working with RNA.

10) Information on special storage conditions (e.g., temperature and humidity, lighting, etc.) and/or user handling of the reagent kit

Storage conditions

Store the RT-PCR Tropic-test-Classic reagent kit (configuration form 1) and RT-PCR Tropic-test-Cito reagent kit (configuration form 2) included into the RT-PCR Tropic-test reagent kit complectation in manufacturer's packaging at -18°C...-22°C during the entire shelf-life period. It is allowed to store the kit at +2°C...+6°C up to 30 days. It is allowed to freeze/thaw the reagent kit up to 10 times.

The reagent kit stored under the regulated conditions violation cannot be used.

11) Information on the in vitro diagnostics reagent kit stability characteristics (e.g., storage conditions, shelf life after a primary packaging first opening), as well as solutions storage and stability conditions

Storage.

Store the RT-PCR Tropic-test-Classic reagent kit (configuration form 1) and RT-PCR Tropic-test-Cito reagent kit (configuration form 2) included into the Tropic-test reagent kit configuration in manufacturer's packaging at -18°C...-22°C during the entire shelf-life period. It is allowed to store the kit at +2°C...+6°C up to 30 days. It is allowed to

freeze/thaw the reagent kit up to 10 times.

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

The reagent kit stored under the regulated conditions violation cannot be used.

Transportation.

The Tropic-test reagent kit can be transported by all types of covered vehicles in accordance with the transportation rules applicable for the vehicle type.

Transport the RT-PCR Tropic-test-Classic reagent kit (configuration form 1) and RT-PCR Tropic-test-Cito reagent kit (configuration form 2) included into the Tropic-test reagent kit configuration at -18°C…-22°C during the entire shelf-life period. It is allowed to transport the reagent kit at +2°C...+6°C up to 30 days or at ambient temperature below +25°C up to 2 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. Tropic-test reagent kit shelf life is 12 months from the acceptance date by the manufacturer's Quality Control Department (QCD) at -18°C...-22°C if all the transportation, storage and usage conditions are met. A reagent kit with an expired shelf life cannot be used.

Opened kit components shelf life is 12 months from the acceptance date by the manufacturer's QCD under all the transportation, storage and usage conditions. A reagent kit with an expired shelf life cannot be used.

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

12) Information on sterility, sterilization method and procedure in case of sterile packaging violation

Sterility: the kit is not sterile.

13) Information for users (warnings, precautions, necessary measures and limitations when using an in vitro diagnostic medical device)

Potential risk Class — 3 — in accordance with Nomenclature Risk Classification of Medical Devices approved by EEC dated December 22, 2015 No. 173.

All components and reagents included in the Tropic-test reagent kit belong to hazard class 4 (low-hazard substances) in accordance with GOST 12.1.007-76 "OSSS. Harmful substances. Classification and general safety requirements".

The reagents included in the Tropic-test reagent kit have low vapor pressure and exclude the possibility of inhalation poisoning.

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

Specialists who gave written consent and were instructed by employees of Rospotrebnadzor laboratories who have a sanitary and epidemiological certificate to work with pathogens of human infectious diseases of pathogenicity group II are allowed to work with test systems for Chikungunya, Zika, and Dengue fever diagnostics in laboratories of organizations.

Clinical material sampling and its packaging is carried out by a medical organization employee trained in the requirements and rules of biological safety when working and collecting material suspected of infection with pathogenicity group II microorganisms. Each sample is placed in a separate container for transportation to ensure the requirements of these guidelines.

All the samples collected for laboratory testing should be considered as potentially infectious, and medical personnel who collect or transport clinical samples must strictly comply with the requirements of biological safety as when working with pathogenicity group II microorganisms.

Samples transportation must be carried out in accordance with the requirements of sanitary legislation in relation to microorganisms of the pathogenicity group II.

All the samples obtained for laboratory testing should be considered potentially infected, and the requirements of SanPiN 1.3.3118-13 should be taken into account when working with them

"Safety work with microorganisms of I - II groups pathogenicity". Healthcare workers who collect or transport clinical samples to a laboratory should be trained in safe biomaterial handling practices, strictly follow the precautions and use personal protective equipment (PPE).

Work with material infected or suspected of being infected with Chikungunya virus is carried out in accordance with the requirements of sanitary and epidemiological rules for the safety of working with microorganisms of pathogenicity groups I-II (SanPiN 1.3.3118-13).

It is required to simultaneously ensure and comply with the biological safety rules and requirements for the organization and conduction of these works by personnel in order to prevent premises and equipment contamination with nucleic acids and (or) amplicons of the test samples.

The work should be carried out in a laboratory performing molecular-biological (PCR) testing of clinical material in accordance with SanPiN 2.1.3684-21 "Sanitary and epidemiological requirements for the maintenance of the territories of urban and rural settlements, water bodies, drinking water and drinking water supply, atmospheric air, soils, residential premises, operation of industrial, public premises, organization and implementation of sanitary and anti-epidemic (preventive) measures". Follow the recommendations laid out in MU 287-113.

The following requirements should always be met when working:

– remove unused reagents in accordance with SanPiN 2.1.3684-21 "Sanitary and epidemiological requirements for the maintenance of the territories of urban and rural settlements, water bodies, drinking water and drinking water supply, atmospheric air, soils, residential premises, operation of industrial, public premises, organization and implementation of sanitary and anti-epidemic (preventive) measures";

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

– use the kit strictly for its intended purpose, according to these instructions;

– do not use the kit after the expiration date;

– avoid contact with skin, eyes and mucous membrane. In case of

contact, immediately flush the affected area with water and seek medical assistance.

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

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

14) Information on the intended use of a single-use in vitro diagnostic medical device

The reagent kit is intended for partial single use.

15) Information on the proper treatment of an in vitro diagnostic medical device for reuse, including cleaning, disinfection, packaging, and, if necessary, re-sterilization method (if the reagent kit is intended for repeated use)

Not applicable.

16) Special requirements regarding premises, special training or special qualifications of the user and/or third parties

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).

The work should be carried out in a laboratory performing molecular-biological (PCR) testing of clinical material in accordance with the applicable rules and regulations.

17) Information on the conditions required for samples collection, treatment and preparation, test samples stability data including storage conditions and duration, transportation conditions, limitations on freezing (thawing) cycles

ATTENTION! Before starting work, it is required to study the methodological recommendations "Sampling, Transportation and Storage of Clinical Material for PCR Diagnostics", developed by the FBIS Central Research Institute of Rospotrebnadzor, Moscow, 2012.

Test sample type

When using configuration form 1: RNA samples isolated from blood plasma.

When using configuration form 2: clinical material for the assay is blood plasma (no RNA isolation procedure is required).

ATTENTION! Avoid repeated freezing and thawing of samples.

17.1 Clinical material collection

ATTENTION! Before starting work, it is required to study the methodological recommendations "Sampling, Transportation and Storage of Clinical Material for PCR Diagnostics", developed by the FBIS Central Research Institute of Rospotrebnadzor, Moscow, 2012.

Clinical material sampling, packaging, transportation and labeling is carried out in accordance with the requirements and rules of biological safety when working with materials suspected of infection with pathogenicity group II microorganisms according to MU 1.3.2569-09 "Organization of the working process in laboratories using nucleic acid amplification methods in working with material, containing microorganisms of I–IV pathogenic groups."

Healthcare workers who collect, label and pack the clinical material must be instructed on sanitary and epidemiological requirements and rules of biological safety when working with patients potentially infected with pathogenicity group II microorganisms.

Material sampling for an assay

Sample 4 or 6 ml of peripheral blood on an empty stomach into a tube (vacuum tube) with EDTA-K2 solution used as an anticoagulant. Turn the tube upside down several times after sampling blood to thoroughly mix blood with EDTA-K2 solution.

ATTENTION! It is not allowed to use heparin and sodium citrate as anticoagulant.

ATTENTION! Heparin presence in blood of patients undergoing anticoagulant therapy can lead to unreliable PCR results, therefore, it is recommended to take blood from such patients before the next administration of the drug.

Transportation and storage conditions of the initial clinical material — blood:

– at +2°C...+8°C — up to 6 hours;

– at room temperature — up to 2 hours. Do not freeze blood.

Plasma should be taken within 2 hours (if stored at room temperature) or 6 hours (if stored at +2°C...+8°C) after the material sampling by centrifuging the tube with blood at 800-1600 g for 20 minutes at room temperature. Transfer the upper fraction (plasma) after centrifugation into separate plastic 1.5 or 2.0 ml tubes free from DNases and RNases.

Blood plasma transportation and storage conditions:

It is allowed to store blood plasma

at +2°C...+8°C — up to 5 days;

at -18°C... -22°C — up to 3 months;

at -70°C — for a long time.

ATTENTION! Avoid repeated freezing and thawing of blood plasma samples.

Use at least 100 µl of plasma to isolate NA. The reagent kit analytical sensitivity may increase due to use of a larger plasma volume, if it is allowed by the used NA isolation reagent kit as well as due to reducing the elution volume.

17.2. Clinical material preparation

Sample preparation in accordance with the used reagent kit for nucleic acids isolation.

Add 10 µl of ICS to 100 µl of blood plasma during nucleic acids isolation.

Configuration form 2 does not require any preparation.

18) Detailed information on the in vitro diagnostic medical device preparation for use

It is not required to install, adjust and calibrate the reagent kit for commissioning.

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

ATTENTION! Mix the reaction mixture components right before the assay.

Reagent kit components preparation for testing

1. Mix thoroughly the tube contents with the isolated RNA, NC, Primer-mix, RT-PCR-buffer 5x, PC turning each tube over 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. Take the required number of tube strips or tubes for RNA test and control samples amplification.

Before RT-PCR reaction conduction 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.

19) Information required for the reagent kit proper installation and readiness for safe operation verification according to the purpose determined by the manufacturer

Not applicable.

20) Recommendations regarding quality control procedures (if needed)

Not applicable.

21) Information on the values traceability set for calibrators or control materials that is provided by reference measurement methods and/or standards.

Metrological traceability of the values attributed to calibrators and control materials in relation to reference method: DNA concentration evaluation is carried out by spectrophotometric method during a PC preparation followed by an amplification reaction for metrological traceability of a positive control sample (PC) included in the kit.

22) Testing procedure including test result calculations and interpretations and information on advisability of confirmatory tests;

PCR procedure consists of the following stages:

A) RNA isolation (if using configuration form 1);

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

C) Results registration and interpretation.

A) RNA isolation from clinical material

(only when using Tropic-test-Classic reagent kit (configuration form 1));

It is recommended to use the following reagent kits for human NA

isolation from blood plasma:

- NA-Extra reagent kit for DNA/RNA isolation from clinical material according to TS 21.20.23-013-97638376-2019, manufactured by TestGene LLC, Russia (registration certificate No. RZN 2021/15428 dated September 24, 2021);

- Reagent kit for nucleic acids extraction (PREP-NA/PREP-NA-PLUS) according to TS 9398-035-46482062-2009, manufactured by DNA-Technology, Russia (registration certificate No. FSR 2010/08867 dated October13, 2016);

or similar ones, designed to isolate RNA from blood plasma and to ensure the isolated RNA quality

- RNA isolation purity, expressed in terms of optical densities (A260/280nm): at least 1.6;

- RNA isolation efficiency: at least 20%.

Strictly follow the protocol and the instructions of the used reagent kit during the RNA extraction procedure.

Add 10 µl of ICS from Tropic-test-Classic reagent kit to each plasma test sample before isolation.

100 µl of NC also undergoes the NA extraction procedure without adding ICS. If the reagent kit manufacturer's instructions require to use a larger sample volume for NA isolation, increase the NC volume to the required volume with saline or TE-buffer.

NA samples storage conditions:

In accordance with the instructions of the used NA extraction reagent kit.

B) RNA RT-PCR with hybridization-fluorescence detection of amplification products in real time;

RT-PCR conduction for Tropic-test-Classic (configuration form 1)

RT-PCR kit components preparation

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 before preparing the reaction mixtures.

1. Mix thoroughly the tubes contents with the isolated for test RNA, RT-PCR-buffer 5x, Primer-mix, NC after isolation, PC, 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. Take the required number of 0.1 or 0.2 ml PCR tubes (with optically transparent lids or walls – depending on the used cycler) according to the following calculation: test samples number + PC + NC.

To prepare a reaction mixture:

1. RT-PCR-buffer 5x – 5 µl,
2. Primer-mix – 5 µl,
3. Sample (RNA test sample, PC, NC) – 15 µl.

Total reaction volume is 25 µl.

ATTENTION! It is forbidden to change the reaction volumes.

RT-PCR protocol

Prepare the reaction tubes in the following order:

1. Label 0.1 or 0.2 ml PCR tubes.

2. In a separate disposable sterile 1.5 or 2.0 ml Eppendorf type tube prepare a reaction mixture: (n+3)x5 µl of PCR-buffer 5x (n+3)x5 µl of Primer-mix, where n is the number of test samples. Mix thoroughly the reaction mixture for 3-5 minutes using vortex.

3. Add 10 µl of the reaction mixture into the corresponding prepared PCR tubes.

4. Add 15 µl of extracted RNA into the corresponding tubes. Do not add RNA into the PC and NC tubes.

5. Add 15 µl of PC into the corresponding tube.

6. Add 15 µl of NC after NA isolation into the corresponding tube.

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

8. Load the PCR tubes into a reaction module of a real-time PCR device. It is recommended to install the tubes in the center of the thermal block to ensure that the tubes are pressed evenly by the heating lid.

9. Program the device to perform the corresponding RT-PCR and fluorescence signal detection programs according to the instructions for the used device (Table 4).

ATTENTION! In case of using QuantStudio 5 cycler it is necessary to adjust optical filters before the amplification protocol conduction (the VIC signal may be registered in the ROX channel if ΔRn

> 100 000, it may lead to obtaining false positive results)! To do that press "Action" button in the "Method" tab, and then in the pop-up window select "Optical filter settings". In the "PCR Filter" tab set only the following filter combinations: x1 – m1, x2 – m2, x4 – m4, x5 – m5, x6 – m6.

Table 4 – RT-PCR protocol

Stage	Temperature, °C	Time, min.:sec.	Detection channels	Total cycles
1	52	25:00	–	–
2	95	02:00	–	–
3	95	00:15	–	5
	64	00:20		
4	95	00:15	–	45
	64	00:20	FAM/Green, ROX/Orange, Cy5/Red, HEX/Yellow	

Specify the samples number and identifiers, mark the tubes location on the thermal lock matrix in accordance with their layout.

10. Make sure that the FAM/Green, ROX/Orange, Cy5/Red, HEX/Yellow detection channels are applied for the amplification program optical measurement parameters.

11. Start RT-PCR with a fluorescent signal detection.

12. Upon the program completion start analyzing the results.

RT-PCR reaction conduction for Tropic-test-Cito (configuration form 2)

RT-PCR kit components preparation

1. Thoroughly mix the test tubes contents with the prepared clinical material, NC, RT-PCR-buffer-Cito 5x, ICS, Primer-mix, and PC turning each tube 10 times or mix using vortex at low speed for 3-5 seconds, then remove the drops from the tube lids by short centrifugation.

2. Take the required number of 0.1 – 0.2 ml RT-PCR tubes according to the number of test samples, PC and NC.

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.

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

Each reaction preparation requires:

To prepare a reaction mixture:

1. RT-PCR-buffer-Cito 5x – 5 µl;
2. Primer-mix – 5 µl,
3. Sample (test sample – 5 µl with 10 µl of ICS addition, PC or NC) – 15 µl.

Total reaction volume is 25 µl.

RT-PCR protocol

Prepare the reaction tubes according to Table 5 in the following order:

1. Label 0.1-0.2 ml RT-PCR tubes: one for each test sample[2], one for PC and one for NC.

Table 5 — Example of tubes layout and PCR components adding

Component	Sample 1		Sample 2		Control samples	
	repetitions		repetitions		PC	NC
RT-PCR-buffer-Cito 5x, µl	5	5	5	5	5	5
Primer-mix, µl	5	5	5	5	5	5
Sample, µl	5	5	5	5	-	-
ICS, µl	10	10	10	10	-	-
PC, µl	-	-	-	-	15	-
NC, µl	-	-	-	-	-	15

2. Add 5 µl of RT-PCR-buffer-Cito 5x into each tube.
3. Add 5 µl of Primer-mix into each tube.
4. Add 5 µl of the sample into the corresponding tubes for test samples. Do not add the sample into the PC and NC tubes.
5. Add 10 µl of ICS into the corresponding tubes. Do not add ICS into the PC and NC tubes.
6. Add 15 µl of PC into the corresponding tube.
7. Add 15 µl of NC into the corresponding tube.
8. Centrifugate the tubes for 1-3 seconds to remove the drops from the walls. Use a microcentrifuge-vortex.

[2] It is recommended to analyze each sample in two repetitions to improve accuracy.

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

10. Program the device to perform the corresponding amplification program according to the instructions for the used cycler. RT-PCR protocol is specified in Table 4.

ATTENTION! In case of using QuantStudio 5 cycler it is required to adjust optical filters before the amplification protocol conduction (the VIC signal may be registered in the ROX channel if ΔRn > 100 000, it may lead to obtaining false positive results)! to do this click the "Action" button in the "Method" tab, and then in the pop-up window chose "Optical filter settings". In the "PCR Filter" tab set only the following filter combinations: x1 – m1, x2 – m2, x4 – m4, x5 – m5, x6 – m6.

11. Specify the samples number and identifiers, mark the tubes location on the thermal block matrix in accordance with their layout.

12. Make sure that the FAM/Green, ROX/Orange, Cy5/Red, HEX/Yellow detection channels are applied for the amplification program optical measurement parameters.

13. Start RT-PCR with a fluorescent signal detection.

14. Upon the program completion start analyzing the results.

C) Results registration and interpretation

Results registration is carried out automatically during amplification via 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 10-20% of the maximum fluorescence level registered for a positive control sample in the last amplification cycle.

The results are interpreted using the FAM/Green, ROX/Orange, Cy5/Red, HEX/Yellow channels Ct values (Table 1). Only Ct values obtained during PCR with fluorescence detection are taken into account (i.e. corresponding to stage 4 — see Table 4).

The reaction rate and Ct values in control samples are evaluated first. Results interpretation is carried out only after correct PC and NC reactions.

If you use Rotor-Gene 6000, Rotor-Gene 3000, Rotor-Gene Q and similar cyclers activate the "Dynamic Tube" and "Noise slope correction" functions, set 10% for "Outlier Removal".

In case of using CFX 96 cycler (BioRad) set the Ct analysis range from 5 to 45 or from 10 to 45 cycles to correct the baseline.

Results interpretation in control samples

The following results should be obtained for negative and positive control samples (Table 6).

Table 6 – PC and NC assay results

Added material	Selected fluorophore			
	FAM (Dengue virus RNA)	ROX (Zika virus RNA)	Cy5 (Chikungunya virus RNA)	HEX (ICS)
NC	> 40 or absent	> 40 or absent	> 40 or absent	> 40 or absent
PC	Ct ≤ 30	Ct ≤ 30	Ct ≤ 30	Ct ≤ 32

Note: "absent" means there is no Ct.

When obtaining NC values that differ from those mentioned in Table 6, the entire assay batch results are considered unreliable. In this case take special measures to eliminate possible contamination.

If obtained PC values differ from those mentioned in Table 6, it is required to repeat amplification of the entire sample batch. If after repeated amplification obtained PC results differ from those mentioned in Table 6, the reagents must be replaced.

Results interpretation in RNA test samples

Results interpretation principle is shown in Table 7.

Table 7 – Results interpretation principle

Ct values in the detection channels corresponding to fluorophores (target analytes are indicated in brackets)				Result
FAM/Green (Dengue virus RNA)	ROX/Orange (Zika virus RNA)	Cy5/Red (Chikungunya virus RNA)	HEX/Yellow (ICS)	
-	-	-	Ct ≤32	Dengue, Zika and Chikungunya virus RNA is **not detected**
Ct >40 or absent			Ct>32 or absent	invalid result
Ct ≤40			not considered[3]	virus RNA **is detected** for a target corresponding to a channel
Ct >40			Ct ≤32	result is doubtful for a target corresponding to a channel

Reason for obtaining an invalid result may be a low NA concentration, inhibitors presence in NA isolated from clinical material; deviation from the assay protocol; the RT-PCR temperature regime violation and etc.

Low virus concentration may be a reason for obtaining a doubtful result.

If the result is invalid the conclusion is not issued. It is required to re-take the biomaterial from the patient and retest it.

23) Analytical efficiency characteristics

23.1 Analytical specificity

Specific to Dengue (serotypes 1-4), Zika, and Chikungunya virus RNA.

Cross-reactivity absence was shown *in vitro*: human

[3] At high concentrations of Dengue, Zika, and Chikungunya virus RNA, HEX channel output may occur in late cycles or be absent.

immunodeficiency virus 1 (HIV-1), human immunodeficiency virus 2 (HIV-2), hepatitis B (HBV), hepatitis C (HCV), hepatitis D (HDV), *Plasmodium*; in *silico*: *Bartonella quintana*, *B. henselae*, *Borrelia bisetti*, *B. garinii*, *B. japonica*, *B. spielmanii*, *Coxiella burnetii*, *Dobrava-Belgrade orthohantavirus*, Japanese Encephalitis virus, *Leptospira interrogans*, *L. kirshneri, L. borgpetersenii*, Puumala orthohantavirus, *Rickettsia conorii*, *R. hejlonjiangensis*, Tick Borne Encephalitis Virus (TBEV), *Treponema pallidum*, *Trypanosoma cruzi*, West Nile virus, and Yellow Fever virus.

23.2 Analytical sensitivity

Configuration form 1: at least 500 copies of Chikungunya, Zika, Dengue virus RNA per 1 ml of clinical material.

Configuration form 2: at least 1000 copies of Chikungunya, Zika, Dengue virus RNA per 1 ml of clinical material.

23.3 Precision under repeatability conditions

To assess precision under repeatability conditions PC and ICS were examined in the four detection channels (FAM, ROX, Cy5, HEX) in 10 repetitions.

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

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

According to the assay results the variation index under repeatability is not higher than 5%.

23.4 Precision under reproducibility conditions

The test system reproducibility evaluation is carried out similarly to the precision under repeatability conditions evaluation (see Section 23.2). However, different batches of the reagent kit were used for testing and testings were 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).

Intra-assay, inter-assay and inter-series reproducibility were performed when conducting precision testing under reproducibility conditions, variation index was not higher than 5%.

23.5 Detection limit (detections)

Configuration form 1: at least 500 copies of Chikungunya, Zika, Dengue virus RNA per 1 ml of clinical material.

Configuration form 2: at least 1000 copies of Chikungunya, Zika, Dengue virus RNA per 1 ml of clinical material.

24) Clinical efficiency characteristics: diagnostic sensitivity and diagnostic specificity

Test sample type	**Number of observations**	**Diagnostic sensitivity**	**Diagnostic specificity**	**Confidence interval** with 95% confidence coefficient
Blood plasma		100%	100%	

25) Biological reference interval

Not applicable.

26) Information on interfering substances or limitations related to a sample that may affect the assay result

The potentially interfering substances effect on the Tropic-test reagent kit performance was examined for potentially interfering substances that may be found during clinical material sampling in the following concentrations:

for configuration form 1:

Interfering substances:

- hemoglobin – 10%;
- heparin (anticoagulant) – 0.15 IU/ml;
- sodium citrate (anticoagulant) – 0.1 mM/ml;
- EDTA-K2 (anticoagulant) – 0.5 mM/ml;
- cholesterol – 150 mg/dL;
- triglycerides – 250 mg/dl;

for configuration form 2:

- hemoglobin – 0.1%;
- heparin (anticoagulant) – 0.0015 IU/ml;
- sodium citrate (anticoagulant) – 0.1 mM/ml;

– EDTA-K2 (anticoagulant) – 0.5 mM/ml;
– cholesterol – 150 mg/dL;
– triglycerides – 250 mg/dL.

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

Limitations on test material use:

– it is not allowed to use test material under storage and transportation conditions violation (temperature, duration, multiple freezing-thawing);

– it is not allowed to use samples contaminated with extraneous biological material.

27) Warnings and/or special precautions regarding the in vitro diagnostics medical device and its accessories safe disposal (if included)

The reagent kit does not emit harmful substances that pollute the environment during storage and transportation. Waste, originated from using a reagent kit for the intended purpose specified by the manufacturer, as well as unused kits (expired shelf life, damaged consumer packaging/labeling, damaged reagent packaging/labeling and etc.) are classified as medical waste.

Medical waste must be collected, neutralized, placed, stored, transported, accounted and disposed in accordance with applicable rules and regulations.

The Tropic-test 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 conducting one or another destruction method.

28) Warnings and/or special precautions regarding a reagent kit intended for self-testing or near-patient testing.

Not applicable.

29) Information on the last issue or the last version of the instructions for use.

30) Information on the need to contact a manufacturer or its authorized representative about undesirable events, having signs of an adverse event (incident)

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

Labelling symbols

Symbol	Symbol name
	Use before
	Manufacture date
	Batch code
	Refer to the instructions for use
	Temperature range
	The content is sufficient for 94 detections