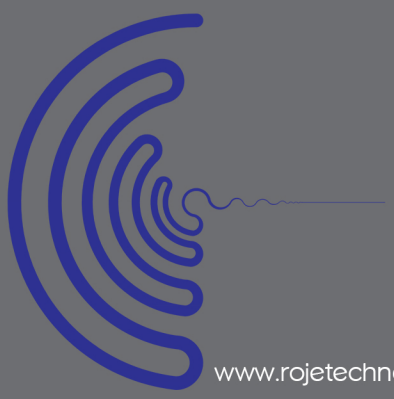

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ROJETECHNOLOGIES

Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit,

One-step Multiplex RT-PCR Assay

SARS-COV-2& Influenza Diagnostic Kit based on Real-Time PCR

For In Vitro Diagnostic Use

BY ROJE

Edition, 02 /2023

ROJETechnologies has been founded since 2014, and manufactures a wide range of molecular biology kits. We research, develop and create our products in order to make easier and more comfortable approaches to do research in molecular biology. Our target is offering high-quality affordable Molecular and diagnostic Kits and reagents, comparable of the world leaders, to research centers, laboratories, clinics, hospitals and diagnostic centers all over the world.

Contents

Kit content

Viga SARS-CoV-2 and Influenza A/B Molecular Diagnostic Kit

Components	100 Preps
Q-ROMAX, 4X	500µl
Pro II Mix	400µl
RTase, Recombinant Reverse Transcriptase, RNase H-(200 U/µl)	100µl
Positive Control	150µl
Negative control	150µl

storage

ROJETechnologies check shipment conditions. After arrival, all reagents should be kept in darkness, at -20 ±5ºC. Do not Freeze-thaw the Kits frequently. When storage conditions are as directed, all reagents are stable until the expiration date, as indicated on the kit box.

Intended use

Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit is a laboratory diagnostic Kit based on the RT-PCR Reverse Transcriptase reaction. This kit diagnoses the N gene of SARS-COV-2, Matrix Protein gene of the Influenza A Virus, and NS1 gene of Influenza B Virus in patients with or suspected infection to SARS or Influenza Virus. Suitable samples were taken from the upper and lower respiratory tract (nasopharyngeal swab, throat swab, interior swab, nasal wash, nasal aspiration, sputum, and bronchoalveolar lavage). These samples are taken from patients or health care staff with COVID-19 or Influenza symptoms.

Guarantee and Warranty

ROJETechnologies guarantee the efficiency of all manufactured kits and reagents. For more information on choosing proper kits based on your needs, please contact our technical support team. If any of the products do not meet your satisfaction, please contact our technical support team due to reasons other than misuse, If the problem is for the manufacturing process, the ROJE team will replace the Kit for you.

Notice to purchaser

This product is only for experimental and not for commercial use. There is no right to resell this kit or any of its components. For information about our licensing or distributors, contact the ROJE business team.

Warning and precautions

- The product is to be used by personnel specially instructed and trained for handling RT-PCR diagnostic cases.
- The Real-Time -PCR instrument requires periodic maintenance and repair.
- Clean tables and appliances periodically.
- Use RNase and DNase-free sterile filter pipette tips.
- Always treat samples as infectious and (bio-)hazardous by safe laboratory procedures, and wear powder-free latex gloves when handling kit material and components.
- To sample, Prepare and perform the reaction, use a separate workstation.
  In each of these workstations, separate tools and equipment must be allocated, and the direction of airflow should be from pre-PCR to post-PCR.
- When using samples with high virus titers and positive control, be careful to avoid contamination of the laboratory environment.
- Change your gloves after handling samples or positive control. Keep your positive control or high load samples separate from other reaction materials.
- Avoid contaminating your workstation materials and equipment with DNA/RNA and nuclease.
- The quality of the isolated RNA affects the quality of the test. Make sure that the RNA isolation method is compatible with the RT-PCR reverse transcriptase technique.
- To eliminate false-positive results due to viral RNA contamination during isolation and ensure the accuracy of results, a negative control (using water instead of sample) should be used at the time of isolation and tested for PCR.
  Optional: use a negative control sample (use water instead of sample) in each PCR reaction.
- Please note the expiration date of the Kit.
- Avoid repeated freezing and thawing of the Kit components; keep Kit components away from light.
- To dispose of samples, follow laboratory waste disposal safety regulations.

Quality control

According to the Clinical and Laboratory Standards Institute and WHO, VIGA SARS-COV-2 Molecular Diagnostic Kit is tested against predetermined experiments on a lot-to-lot basis to ensure consistent product quality. For your information, the results of all experiments are accessible by addressing REF and Lot number on the web at www.rojetechnoloes.com.

Procedure

Viga SARS-COV-2 Molecular Diagnostic kit is a test of One-step Multiplex Real-Time RT-PCR. This kit is designed for the qualitative diagnostic N gene of SARS COVID-19, the M2 gene of Influenza A virus, and the NS1 gene of Influenza B. After viral nucleic acid isolation by using RNJia Virus Kit (Cat No: RN983072) or other kits that the Ministry approve of Health, the combination of the sample can be added to the master mix primer/probe mix and RTase enzyme to perform the reaction. Internal control targets the human RNase gene by which quality of sample collection, nucleic acid isolation, and the whole process of RT-PCR reaction can be checked and controlled to avoid false-negative results. The lowest sensitivity of the kit is 200 copies per ml for Coronavirus and 150 copies per ml for Influenza A and B, respectively.

Types of controls

Negative control: A "no template" (negative) control is used to monitor whether there is contamination for the RT-PCR process and is used in each detection run.

Positive control: A positive template control is used to monitor whether the RT-PCR process works properly and is used in each detection run.

Internal control (RNase P): An internal control for RNase P gene is used to monitor the sample collection, handling and RT-PCR process and is used in each sample amplification.

Equipment and reagents to be supplied by the user

- You need a Nylon or Dacron swab with an aluminum or plastic shaft for sampling.
- DNase-RNase-free microtubes (1.5ml).
- PCR microtube 0.2- or 0.1-ml strip.
- Various pipette and pipette tips (10µl, 100µl, and 1000µl of filter pipette tips).
- Surface sanitizing solution like RNZO (Cat No: RN983048).
- Disposable Powder- Free gloves and surgical gown.
- Different types of Real-Time PCR Instruments (green, yellow, orange, and red channels).
- Centrifuge (which can reach 13000 rpm).
- Microcentrifuge
- Vortex
- Cool box

Real-Time PCR devices

This kit can be run on the following devices.

- Rotor-Gene Q, 5plex
- Corbett Rotor-Gene 3000&6000
- Mic qPCR Cycler
- ABI Step One & Step One Plus
- Biorad CFX96 Real-Time PCR
- Roche LightCycler® 96 Real-Time PCR System
- Anatolia Montania 484 Real-Time PCR Instrument

Tip: more information about the Real Time-PCR device can be read in the device manual template. Notice that some PCR devices must be calibrated with the desired colors before multiplex-PCR reaction.

Applications

The Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit is based on a one-step RT-PCR reverse transcriptase reaction. Part of the RNA sequence of Pathogen is the template for generating cDNA and then used as a PCR reaction model. The resulting PCR product is identified by an oligonucleotide probe that is labeled with fluorescent color. This Kit detect the N gene from SARS-COV-2 (Coronavirus) and the M2 gene from the Influenza A and NS1 gene from the Influenza B virus. Other Coronaviruses and other strains of the Influenza virus are not identified with this Kit.

The area selected for designing the Primer/probe of the N gene provides a conserved region among all SARS-COV-2. N gene of COVID-19 in orange Fluorescence channel is identified in selected areas for M gene of Influenza A virus in green fluorescence channel, and NS1 gene of Influenza B virus in yellow fluorescence channel. These genes are identified in the FAM, Yakima yellow, and Texas Red fluorescence channels for targeting COVID-19, Influenza A, and B, respectively. Internal control or RNase P gene is identified in the Cy5 channel. It is also used as a control to isolate RNA and check the RT-PCR reaction's inhibition. This test is compatible with Real Time-PCR devices that identify four green, yellow, orange, and red fluorescence channels.

Tip: Notice that some Real-Time PCR systems must be calibrated to the appropriate color before performing multiplex PCR.

Sample storage and preparation

Specimen collection

Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic kit is a simultaneous diagnostic kit of viral nucleic acid SARS-COV-2 and Influenza A/Influenza B/ in respiratory samples. All

samples must be contamination-free in sampling, storage, and transportation stages. Consider all samples potentially infectious and transfer the samples by following the biosafety guidelines completely. The collection swab should have a synthetic tip, such as nylon or Dacron, and an aluminum or plastic shaft. A cotton swab with wooden shafts is not recommended at all. After sampling, swabs should be stored in a virus transport medium immediately.

storage and delivery of specimens

it should be tested within 24 hours if the specimen is stored at 4ºC. Samples that cannot be tested within 24 hours should be stored at -70ºC or below (in the absence of -70ºC storage condition, specimens can be stored at -20ºC for ten days, the nucleic acid can be stored at -20±5ºC for 15 days). Multiple freeze-thaw cycles must be avoided.

Specimen isolation

For viral nucleic acid isolation, use RNJia Virus Kit (Cat No: RN983072) or other diagnostic kits approved by the Ministry of health.

Pathogenicity

Coronaviruses are positive single-stranded RNA viruses from the Coronaviridae family. HCOV-229E, HCOV-NL 63, HCOV-OC43, MERS-COV, and HCOV-HKU1 Strains cause cold, upper respiratory tract infections, bronchiolitis, and pneumonia in humans. SARS-COV-2 is a beta coronavirus that appeared in December 2019 in Wuhan. This virus is the cause of COVID-19 disease. Fever, cough, and respiratory problems are the most common early symptoms of the disease leading to pneumonia and severe respiratory syndrome (SARS). Close contact with respiratory droplets transmits the Coronavirus.

Influenza is an acute infectious disease caused by Influenza A and B viruses or, to a lesser extent, Influenza C viruses. The genome of influenza viruses consists of a segmented RNA strand with a negative head located in a protein capsid. These viruses are typical all over the world. The spread of influenza A is mainly due to antigenic drift of hemagglutinin and neuraminidase molecules. Influenza virus types B and C are almost human pathogens, while Influenza A viruses can infect a wide range of warm-blooded animals.

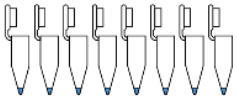
Work station preparation

All work surfaces, pipettes, centrifuges, and other supplies must be cleaned and sanitized. To reduce the risk of nucleic acid contamination, use sanitizers like 70% Ethanol or 10% Sodium Hypochlorite.

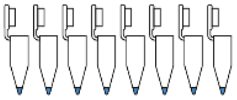
Protocol

Step 1:
Isolated nucleic acid

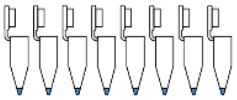
Step 2:
Add molecular biology grade water



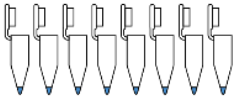
Step 3:
Add Q-ROMAX, 4



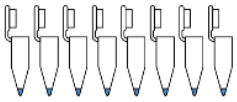
Step 4
Add pro I mix



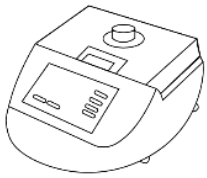
Step 5:
Add RTase enzyme



Step 6:
Add isolated nucleic acid



Step 7:
Run the PCR program



Step 8:
Interpret the PCR results

Results *	Ct Cy5	Ct Texas Red	Ct Yakima	Ct FAM bhbiuhiusdcsc	
A	-/+	Ct< 40	Ct< 40	Ct< 40	Influ A/B**, COVID-19
A	-/+	-	-	Ct<4 0	Influ A +
A	-/+	-	Ct<4 0	-	Influ B+
A	-/+	Ct< 40	-	-	COVID-19+
R	-	-	-	-	False negative
A	-	-	-	-	Negative control
A	Ct<40	-	-	-	Negative clinical sample

Figure 1: preparation of reagents, the addition of isolated DNA, PCR run, and interpretation of results

process

Take out each component from the diagnostic kit and place them at room temperature. Allow the reagents to equilibrate to room temperature, then briefly vortex each for later use. The volume of the isolated sample in this test should be 10µl. Based on table 1, prepare the reaction components and perform Real Time-PCR according to table 2.

Table1: preparation of reaction components for one reaction.

components	Volume
Q-ROMAX	5µl
RTase, Recombinant Reverse Transcriptase, RNase H-(200 U/µl)	1µl
Pro II Mix	4µl
Isolated RNA	10µl

Table 2: temperature program of one-step Multiple Real Time-RT-PCR

step	time	Temperature	Number of cycles
cDNA synthesis	20min	50ºC	1
Polymerase enzyme activation	1min	95ºC	1
denaturation	10s	95ºC	45 cycles
annealing and extension of nucleic acid and measurement of fluorescence in green, yellow, orange, and red channels	40s	60ºC	

Interpretation of clinical results

- Data analysis for each gene should be performed separately using a manual threshold.
- The threshold for each sample should be in the exponential phase of the fluorescence curves and above any background signal.
- FAM Fluorophore (green) to detect Influenza A, Yakima Yellow Fluorophore (Yellow) to detect Influenza B, Texas Red Fluorophore (orange) to detect COVID-19, and Cy5 Fluorophore (Red) is for the RNase P gene (internal control).

- A negative control is used as contamination control. The magnitude increases of the fluorescence curve in the negative control do not cross the threshold. If Ct is less than 35 (Ct<35), it is considered a possible contamination. Strong signals above 35 in the NTC can be PCR artifacts which, in these cases, the shape of the curve can be considered (the S-shaped curve is typical for a positive result).
- Internal control or RNase P gene should be positive for all clinical specimens at Ct 35 or less than 35, indicating sufficient nucleic acid from the human RNase gene and the sample has acceptable quality.
- Internal control curve or RNase P gene Ct>40 or without Ct indicates low sample concentration or inhibitors in the reaction (recommended that the isolated sample be diluted at least ½). If the test result is not acceptable again during the retest, another new sample should be taken from the patient, and the test must be repeated.
- A positive clinical specimen Ct≤40 for the yellow and orange channels indicate Influenza B, and COVID, respectively, And Ct≤36 for the green channel indicate Influenza A. If any of the above channels are positive and the Red channel (internal control) is not, it is considered a negative sample if only the Red channels are positive otherwise, our result is not valid.
- If the expected positive reaction is not achieved (typical S-shaped curve), the performed test is not acceptable, and repeat the test by following the kit instructions exactly.
- Determine the reason for the failure of positive control and the corrective action, and document the corrective action results.

Table 3: valid control criteria

interpretation	Ct Cy5 channel RNase P	Ct Texas Red channel SARS Coronavirus	Ct YakYel channel Influenza B	Ct FAM channel Influenza A	Control
Acceptable	Negative	Ct<40	Ct<40	Ct<36	Influenza A/B and positive COVID19
Unacceptable	Negative	Negative	Negative	Negative	False negative

Acceptable	Negative	Negative	Negative	Ct< 36	Positive Influenza A
Acceptable	Negative	negative	Ct< 40	Negative	Positive Influenza B
Acceptable	Negative	Ct< 40	Negative	Negative	Positive COVID19
Acceptable	Negative	Negative	Negative	Negative	Negative control (water)
Acceptable	Ct<35	Negative	Negative	Negative	Negative extraction control

Test Limitation

The optimal performance of this test also depends on how the sample is collected, transferred, and stored. The present kit is suitable for diagnosing target viruses in swab samples and respiratory sputum. Depending on the type of COVID-19 disease and influenza, the test is performed on samples of people who have the disease symptoms; these samples are collected by the patient itself or by health care staff. A negative test does not reject the possibility of SARS-COV-2, Influenza A, or B virus because test result may be affected by sample collection, user error, how the sample is mixed, or low virus rates that can be less than the sensitivity of the Kit and the presence of PCR inhibitors can cause false-negative results. In general, sequence diversity in the target area of unknown viruses may lead to false-negative results or less Kit sensitivity. The results should be interpreted based on clinical findings and other tests.

Performance evaluation

selection of target gene and primer/probe design

First, sequence data for Influenza A and Influenza B, and Coronavirus 2 were obtained from NCBI archives. After setting out the databank for each of the target genes, bioinformatics software such as Mega ten and Claustral were used for alignment analysis, and the most conserved region was selected for designing primers/probes by Beacon Designer and AlleleID 7.5 software. This Kit N gene of the SARS-COV-2, M2 gene of Influenza A, and NS1 gene of Influenza B were used to design the primer and probe; after designing the primer and probe, Oligo7 and Gene runner, NCBI blast evaluated their specificity and characteristic.

Preparation of a standard sample

RNA sample with a (concentration of 200000 copies per ml) for SARS-COV-2 and RNA sample with (a concentration of 15000 copies per ml) were prepared AMPLIRUN® INFLUENZA A H1 RNA CONTROL and AMPLIRUN® INFLUENZA B RNA CONTROL controls for Influenza A and B respectively. From the same samples, dilutions of 1/10, 1/100, and 1/1000 were prepared (to dilute the sample, dilute it to a ratio of 110. This was done by diluting 100µl of the initial sample with 900µl of water). An average of six repetitions with Novel Coronavirus (2019-nCOV) Nucleic Acid Diagnostic kit (Sansure Biotech Inc) for diagnosing Coronavirus and from RealStar®Influenza Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics GmbH) kit for diagnosing Influenza A and B. The results are shown in the table below.

Table 4: Average of Ct six repetition for N gene in 2019-ncov positive specimen

Test for 2019- nCoV Using (Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (Sansure Biotech Inc)	Target gene 2019-nCoV virus	Prepared concentration For testing [copies/ml]	Average of Ct Six repeats
2019-nCoV positive specimen	N gene	200000	26.20668757
		20000	29.63871707
		2000	33.39302301
		200	36.72828386

Table 5: Average of Ct six repetition for identifying candidate gene in Influenza B positive specimen and Influenza A positive specimen

Test for diagnosing Influenza A and B RealStar®Influenza using Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics GmbH)	Prepare concentration for testing [copies/ml]	Average of Ct six repeats
Influenza A positive specimen	15000	28.89753141
	1500	33.35089274
	150	36.73464453
	15	39.3650118

Influenza B positive specimen	15000	29.39214087
	1500	33.68245458
	150	36.94023701
	15	39.52432607

Then the identical diluted specimens were performed using simultaneous detection of nucleic acid (Viga SARS_COV_2 and Influenza A /B); the results are in the following table:

Table 6: average of Ct six repetition for N, M, and NS1 genes in Influenza A positive specimen, Influenza B positive specimen, 2019-nCOV positive specimen.

Average of Ct Six repetitions were performed using a simultaneous nucleic acid detection Kit (Viga Influenza A/Influenza B/Coronavirus 2 Real-Time RT-PCR Kit)	**Prepared concentration for testing [copies/ml]**	**Target genes**	**test for simultaneous diagnosis using a nucleic acid detection Kit (Viga Influenza A/Influenza B/Coronavirus 2 COVID-19 Real-Time RT-PCR Kit)**
26.22646827	200000	N gene	2019-nCoV positive specimen
30.37436598	20000		
34.49661426	2000		
39.41351	200		
26.14566584	15000	M gene	Influenza A positive specimen
30.20130167	1500		
34.75491914	150		
Undetermined	15		
28.37268058	15000	NS1 gene	Influenza B positive specimen
32.03218378	1500		
35.86144	150		
Undetermined	15		

Limit of Detection (LoD)-Analytical sensitivity

LoD studies were used to determine the lowest detectable concentration of Influenza A/B and Coronavirus 2 RNA, at which approximately 95% of all (true positive) replicates test positive.

Dilution of 200 copies per ml of novel Coronavirus and 150 copies per ml of Influenza A and B were used to determine the minimum limit of detection. 200, 100, and 50 copies of Coronavirus and 150, 75, and 35 copies per ml of Influenza A and B were prepared and placed in 8 repetitions of each dilution. The following results were obtained for each virus. The lowest limit of detection in Viga SARS-COV-2 Molecular Diagnostic Kit is 100 copies per ml. for nucleic acid simultaneous detection Kit (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit Real-Time RT-PCR) 200 copies per ml for Coronavirus and 150ml for Influenza A and B were obtained, respectively.

Table 7: The lowest detectable concentration of Influenza A/ Influenza B and Coronavirus 2 using Viga SARS-COV2 and Influenza A/B.

Test No.	**Concentration (Copies/ml)**								
	N gene (COVID-19)			NS1 gene Influenza B			M gene Influenza A		
	200	100	50	150	75	35	150	75	35
1	39.321	39.865	Undetermined	35.45100934	38.7966	Undetermined	35.10396	37.02104954	Undetermined
2	39.453	39.9734	Undetermined	35.87735	37.82469	Undetermined	34.2054	37.17168956	Undetermined
3	39.7553	40.523	Undetermined	35.55521	41.09085	Undetermined	34.47724	37.11441002	Undetermined
4	39.2552	39.999	Undetermined	35.7325	40.08217	Undetermined	34.40626	37.11701312	Undetermined
5	39.023	Undetermined	Undetermined	35.67410	Undetermined	Undetermined	35.8531	37.18172035	Undetermined
6	39.4231	40.523	Undetermined	36.6342	Undetermined	Undetermined	35.31948325	37.30105653	Undetermined
7	39.753	Undetermined	41.568	36.33275	Undetermined	Undetermined	34.29835607	37.03268394	Undetermined
8	39.3244	Undetermined	42.862	35.63442	Undetermined	Undetermined	34.37555378	Undetermined	Undetermined
Positive percentage in	100%	62.5%	25%	100%	50%	-	100%	87.5%	**-**

each concentration									

Analytical sensitivity

Primer/ probe sets used in simultaneous detection of nucleic acid (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit) in-silico for SARS-COV-2 and Influenza A and B was reviewed at the NCBI site. Alignment analysis of primer/probe sequences for N gene from COVID-19, M gene from Influenza A, and NS1 from Influenza B showed 100% overlap with the sequences of each virus. The alignment results for each gene are shown in the table below.

Table 8: Results of in-silico analysis for primer/probe of Coronavirus (2019-nCOV) N gene against sequences reported at NCBI site.

Strain	Target	Accession	% Homology Test Forward primer%	% Homology Test Reverse primer%	% Homology Test Probe%
SARS-CoV-2/human/TWN/CGMH-CGU-36/2020	N gene	MW356672.1	100	100	100
SARS coronavirus Tor2 isolate Tor2/FP1-10912	N gene	JX163923.1	100	100	100
BetaCoV/Wuhan/WH-01/2019	N gene	CNA0007332	100	100	100
Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/TX-DSHS-1535/2020	N gene	MW349166.1	100	100	100
SARS-CoV-2/Canis familiaris/USA/TAMU-077/2020	N gene	MW263336.1	100	100	100
SARS-CoV-2/human/ECU/Z&Z_SARS_4/2020	N gene	MW294011.1	100	100	100
SARS coronavirus isolate Guangdong/20SF012/2020	N gene	EPI_ISL_403932	100	100	100

SARS coronavirus isolate Xiao Tang Shang Hospital polyprotein 1ab-like gene	N gene	AY465926.1	100	100	100
Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/MN-MDH-2049/2020	N gene	MW349104.1	100	100	100
SARS-CoV-2/human/TWN/CGMH-CGU-36/2020	N gene	MW356672.1	100	100	100

Table 9: Results of in-silico analysis for primer/probe of Influenza A M2 gene against sequences reported at NBCI site.

Strain	Target	Accession	% Homology Test Forward primer%	% Homology Test Reverse primer%	% Homology Test Probe%
Influenza A virus (A/swine/North Carolina/A02478981/2020(H1N1) matrix protein 1 (M1)	M1	MT644554.1	100	100	100
Influenza A virus (A/swine/Iowa/A02478968/2020(H1N2) matrix protein 2 (M2)	M1	MT644542.1	100	100	100
Influenza A virus (A/Texas/9804/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MT639878.1	100	100	100
Influenza A virus (A/South Korea/9793/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MT639798.1	100	100	100
Influenza A virus (A/South Korea/9792/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MT639790.1	100	100	100
Influenza A virus (A/Ohio/9270/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MT638933.1	100	100	100
Influenza A virus (A/Mallard duck/Alberta/471/2019(H3N8)) segment 7 matrix protein 2 (M2)	M1	MT624468.1	100	100	100
Influenza A virus (A/Iowa/59/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MN948660.1	100	100	100
Influenza A virus (A/Oklahoma/24/2019(H1N1)) segment 7 matrix protein 2 (M2)	M1	MN948318.1	100	100	100
Influenza A virus (A/California/196/2019(H1N1)) segment 7 matrix protein 2 (M2)	M1	MN948118.1	100	100	100

Influenza A virus (A/Germany/9203/2019(H3N2)) segment 7 matrix protein 2 (M2)	M1	MT638613.1	100	100	100

Table 10: results of in-silico for primer/probe of Influenza B NS1 gene against sequences reported at NCBI site.

Strain	Target	Accession	% Homology Test Forward primer%	% Homology Test Reverse primer%	% Homology Test Probe%
Influenza B virus (B/West Virginia/17/2019) nonstructural protein 1 (NS1) genes	NS1	MT315925.1	100	100	100
Influenza B virus (B/South Carolina/09/2019) nonstructural protein 1 (NS1) genes	NS1	MT315677.1	100	100	100
Influenza B virus (B/Ohio/04/2020) nonstructural protein 1 (NS1) genes	NS1	MT637903.1	100	100	100
Influenza B virus (B/New York/04/2020) nonstructural protein 1 (NS1) genes	NS1	MT315453.1	100	100	100
Influenza B virus (B/Texas/9813/2019) nonstructural protein 1 (NS1) genes	NS1	MT637911.1	100	100	100
Influenza B virus (B/Montana/01/2020) nonstructural protein 1 (NS1) genes	NS1	MT315253.1	100	100	100
Influenza B virus (B/Minnesota/03/2020) nonstructural protein 1 (NS1) genes	NS1	MT315182.1	100	100	100
Influenza B virus (B/Maryland/01/2020) nonstructural protein 1 (NS1) genes	NS1	MT315101.1	100	100	100
Influenza B virus (B/Kentucky/05/2019) nonstructural protein 1 (NS1) genes	NS1	MT315021.1	100	100	100
Influenza B virus (B/Iowa/37/2019) protein 1 (NS1) genes	NS1	MT314997.1	100	100	100

Clinical sensitivity

Using a negative specimen (pharyngeal and nasal swabs) and a positive specimen, dilution was prepared. The specimen was extracted, and the test using simultaneous detection of nucleic acid kit (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic kit) was repeated three times. The results are in the following table:

Table 11: Result of clinical sensitivity test for simultaneous nucleic acid detection (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic kit).

specimen	Average of Ct NS1 gene (Influenza B)	Average of Ct M gene (Influenza A)	Average of Ct N gene (COVID19)
Considering isolation	28.50515	26.15455	26.24657
	28.10394	26.05473	26.07492
	28.14841	26.13173	26.20971
Regardless of isolation	28.07289	25.82956	25.35822
	28.08063	26.22486	25.52999
	28.01095	25.83337	25.51075

Cross-reactivity (analytical specificity)

This test is for simultaneous detection of nucleic acid (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit) in silico analysis for other respiratory pathogens. Alignment analysis of primer/probe sequences for M2, NS1, and N genes for Influenza A/ B and Coronavirus, respectively, in NCBI database at nr/nt bank using BLASTN 2.10.0+ software, demonstrated that primers/probes detect M2, NS1, and N genes of Influenza A, B, and Coronavirus respectively. Based on this analysis, no cross-reactivity was observed for other respiratory pathogens in the table below.

Table 12: in silico analysis of Coronavirus (2019-nCOV) primer/ probe for other respiratory pathogens.

Pathogen (Taxonomy ID)	Strain	GenBank Acc#	% Homology Test Probe	% Homology Test RP	% Homology Test FP
Influenza A virus	A/Ross's Goose/Arkansas/AH0085761S.4.A/2016(H11N9)	MN253675.1	56	48	40

Influenza B virus	B/Hong Kong/CUHK21967/2000	MF955545.1	52	40	36
MERS-CoV	HKU1 SC2628	MK858156.1	63	54	43
Human coronavirus HKU1	HCoV_OC43/Seattle/USA/SC9428/2018	DQ437612.1	30	36	71
Human coronavirus NL63	-	DQ462758.1	43	36	56
Human adenovirus D8 isolate BA_280-2008 hexon gene	-	MK913814.1	40	60	78
Human coronavirus 229E	camel/Abu Dhabi/B38	MG000870.1	40	36	43
Human coronavirus OC43	KEN/SOK040/2016	MN630561.1	53	56	45
Respiratory syncytial virus	99-727T G	MK634291.1	43	52	73
Haemophilus influenzae	isolate RSVA/USA/ACRI-051/2016	LR739069.1	46	72	60
Mycobacterium tuberculosis	C9_S	AF269311.1	46	76	52
Human Metapneumovirus (hMPV)	MY/U2311/2013	KU320918.1	45	67	62
Parainfluenza virus 1-4	HPIV3/Seattle/USA/7J10/2011	MF973170.1	54	45	48
Enterovirus EVB68	EVB/B78/ETH/GR285/2016	MK815602.1	50	57	63
Respiratory syncytial virus	SC0850	MN306045.1	41	48	56
Rhinovirus	A24 J6-YN-CHN-2017 VP1	LC412982.1	66	53	64
Chlamydia pneumoniae	YK41	LN849050.1	78	65	61
Legionella pneumophila	SBT211	CP045974.1	66	43	57
Streptococcus pneumoniae	2245STDY6092949	LR216055.1	75	57	46
Streptococcus pyogenes	GURSA1	CP022206.1	67	55	57
Bordetella pertussis	A639	CP046993.1	54	45	48

Mycoplasma pneumoniae	16-734	CP039761.1	66	63	57
Pneumocystis jirovecii (PJP)	RU7	XM_018375876.1	43	49	55
Candida albicans	TIMM 1768	CP032019.1	63	58	66
Pseudomonas aeruginosa	PABL017	CP031660.1	49	54	51
Staphylococcus epidermis	O47	CP040883.1	69	46	57
Streptococcus salivarius	NU10	MN480762.1	66	58	53
human genome	AKR1C3	NG_047094.1	70	65	46

Table 13: in silico analysis of (Influenza A) primer/probe for other respiratory pathogens.

Pathogen (Taxonomy ID)	Strain	GenBank Acc#	% Homology Test Probe	% Homology Test RP	% Homology Test FP
Influenza B virus	B/Hong Kong/CUHK21967/2000	MF955545.1	52	58	55
MERS-CoV	HKU1 SC2628	MK858156.1	57	44	48
Human coronavirus HKU1	HCoV_OC43/Seattle/USA/SC9428/2018	DQ437612.1	54	56	65
Human coronavirus NL63		DQ462758.1	63	46	38
Human adenovirus D8 isolate BA_280-2008 hexon gene		MK913814.1	49	56	66
Human coronavirus 229E	camel/Abu Dhabi/B38	MG000870.1	57	44	54
Human coronavirus OC43	KEN/SOK040/2016	MN630561.1	54	38	43
Respiratory syncytial virus	99-727T G	MK634291.1	56	65	58
Haemophilus influenzae	isolate RSVA/USA/ACRI-051/2016	LR739069.1	65	53	54

Mycobacterium tuberculosis	C9_S	AF269311.1	58	53	49
Human Metapneumovirus (hMPV)	HR347-12	KU375602.1	44	57	43
Parainfluenza virus 1-4	HPIV3/Seattle/USA/10I9/2010	MK167037.1	33	64	54
Enterovirus EVA78	EV-G/Pig/JPN/Kana-Ebi6/2019/G8-2/PL-CP	LC549660.1	55	42	58
Respiratory syncytial virus	GH300327/USA/2013	MT422270.1	33	63	49
Rhinovirus	SPb_219/12Hel/NAO-17/14/RU	KU841460.1	55	61	64
Chlamydia pneumoniae	Wien3	LN847257.1	48	59	54
Legionella pneumophila	D-4058	CP021277.1	78	61	66
Streptococcus pneumoniae	2245STDY6178854	LR536841.1	68	56	43
Streptococcus pyogenes	4063-05	CP051138.1	65	61	65
Bordetella pertussis	J029	CP046995.1	56	45	39
Mycoplasma pneumoniae	16-710	CP039762.1	54	58	54
Pneumocystis jirovecii (PJP)	RU7	XM_018375154.1	48	56	49
Candida albicans	TIMM 1768	CP032019.1	75	43	58
Pseudomonas aeruginosa	paerg000	LR130528.1	58	63	67
Staphylococcus epidermis	SESURV_p4_1553	CP043804.1	53	57	61
Streptococcus salivarius	NCTC8618	LR134274.1	62	68	64
human genome	563D14	NG_016276.1	63	48	52

Table 14: in silico analysis of (Influenza B) primer/probe for other respiratory pathogens.

Pathogen (Taxonomy ID)	Strain	GenBank Acc#	% Homology Test Probe	% Homology Test RP	% Homology Test FP
Paramyxoviridae	BtVs-BetaCoV/SC2013	MN938062.1	No Sig.	48	52
Orthomyxoviridae	Human	MF955545.1	45	49	No Sig.
MERS-CoV	HKU1 SC2628	MK858156.1	No Sig.	62	57
Human coronavirus HKU1	HCoV_OC43/Seattle/USA/SC9428/2018	DQ437612.1	67	No Sig.	34
Human coronavirus NL63		DQ462758.1	53	48	No Sig.
Human adenovirus D8 isolate BA_280-2008 hexon gene		MK913814.1	No Sig.	43	45
Human coronavirus 229E	camel/Abu Dhabi/B38	MG000870.1	55	No Sig.	53
Human coronavirus OC43	KEN/SOK040/2016	MN630561.1	33	47	42
Respiratory syncytial virus	99-727T G	MK634291.1	65	64	59
Haemophilus influenzae	isolate RSVA/USA/ACRI-051/2016	LR739069.1	46	72	60
Mycobacterium tuberculosis	C9_S	AF269311.1	46	76	52
Human Metapneumovirus (hMPV)	H0708-164-B	KF179028.1	40	54	47
Parainfluenza virus 1-4	HPIV3/Seattle/USA/SC2288/2015	MF795097.1	63	43	46
Enterovirus EVA76	17-1936-1_A76	MH118024.1	46	53	58

Respiratory syncytial virus	GH300327/USA/2013	MN531557.1	63	52	59
Rhinovirus	NIE0611579	KX162706.1	50	67	56
Chlamydia pneumoniae	YK41	LN849050.1	66	65	73
Legionella pneumophila	D-4040	CP021274.1	60	49	43
Streptococcus pneumoniae	2245STDY6020240	LR536835.1	57	66	53
Streptococcus pyogenes	MGAS6180	CP000056.2	67	53	52
Bordetella pertussis	J299	CP046994.1	57	61	65
Mycoplasma pneumoniae	NCTC10119	LR214945.1	54	55	49
Pneumocystis jirovecii	RU7	XM_018375553.1	40	61	65
Candida albicans	TIMM 1768	CP032019.1	67	53	58
Pseudomonas aeruginosa	NCTC9433	LS483497.1	50	54	46
Staphylococcus epidermis	HD43	CP040867.1	63	64	58
Streptococcus salivarius	ICDC3	CP018189.1	64	53	58
human genome	CH17-240J14	AC275601.1	53	54	61

Cross-reactivity (clinical specificity)

Also, to evaluate the clinical specificity of the nucleic acid of respiratory pathogens such as (Adenovirus, Legionella Pneumophila, Neoformans, Chlamydia Pneumoniae, Streptococcus Pneumonia, Respiratory Syncytial Virus, Mycoplasma Pneumoniae, Streptococcus Pyogenes, Mycobacterium Tuberculosis) in the negative matrix (negative swab specimen) was diluted with a certain concentration. The samples were isolated and tested using simultaneous detection of nucleic acid (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit); no cross-reactivity was observed for the respiratory pathogens in the following table.

Table 15: evaluating the cross-reactivity of novel Coronavirus (2019-nCOV), Influenza A and B using simultaneous nucleic acid detection (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit).

Virus/Bacteria/Parasite	Source/ Sample type	Concentration	Ct Value (ORF1ab gene/N gene)
Adenovirus	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Legionella pneumophila	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Cryptococcus neoformans	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Chlamydia pneumonia	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Streptococcus pneumoniae	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Respiratory Syncytial Virus	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Mycoplasma pneumoniae	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Streptococcus pyogenes	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
Mycobacterium tuberculosis	AmpliRun®DNA/RNA Vircell	10^4 copies/ml	**-/-**
10 Pooled human genomes	Clinical sample	10 ng/µl	**-/-**

Accuracy

Accuracy assessment includes In Vitro Intra-assay and Inter-assay.

Intra-assay

Intra-assay refers to the accuracy and ability of the designed method in determining the concentration of similar repeats in one Real Time-PCR cycle shown as SD for different Cts. For this purpose, three repetitions of each concentration of the control sample were examined in one reaction, and coefficient of variation values was calculated for the threshold cycle values. The Run for Coronavirus (N gene) results in the maximum coefficient of variation is 0.63. The minimum coefficient of variation is 0.20, and for Influenza A (M gene), the maximum coefficient of variation is 2.5. The minimum coefficient of variation is 0.36, for the Influenza B virus (NS1 gene), the maximum coefficient of variation is 1.8, and the minimum coefficient of variation is o.73. All acceptable results must have a cv of less than 5%.

Inter-assay

Inter-assay refers to the results of different Runs in Real Time-PCR or results of other laboratories, usually expressed as SD or CV for different Cts related to the number of copies or different concentrations of a sample. For this purpose, Real Time-PCR reactions, at least five repeats of each concentration of the control sample, were tested on three other days. The result for Coronavirus (N gene) in maximum coefficient of variation is 4.4. The minimum coefficient of variation is 0.8. For the Influenza A virus (M gene), the maximum coefficient of variation is 3/3. The minimum coefficient of variation is 1.75. For Influenza B (NS1 gene) the maximum coefficient of variation is 4/08. The minimum coefficient of variation is 0/06. All acceptable results must have a CV of less than 10%.

Clinical evaluation

The clinical function of simultaneous detection of nucleic acid (Viga SARS-COV-2 Molecular Diagnostic Kit) using 100 negative samples, 110 positive COVID-19 samples, 30 positive Influenza B samples, and 30 positive Influenza B of the throat and nasal swabs (in the Virus transport medium) that collected from patients with suspected COVID-19 were evaluated. To compare and verify the simultaneous detection of nucleic acid (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit) using two Kits Novel Coronavirus (2019-nCoV) Nucleic Acid Coronavirus Diagnostic Kit (PCR Fluorescence Probing) (Sansure Biotech Inc) and RealStar®Influenza Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics GmbH) which were licensed for emergency use in the laboratory by the US food and Drug Administration. Results of clinical assessment for COVID-19 (negative percent agreement) NPA 100% and (positive percent agreement) PPA 97/27% are shown in the table below.

Table 16: clinical assessment between simultaneous detection of nucleic acid Novel Coronavirus, Sansure Biotech, and ROJETechnologies.

Test		**Novel Coronavirus Nucleic Acid Diagnostic Kit (PCR Fluorescence Probing) (Sansure Biotech Inc)**		**Total**
		Positive	Negative	
Simultaneous detection kit (Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit)	Positive	107	0	107
	Negative	3	100	103
Total		110	100	210

- Positive Agreement Rate: 107/110 ×100%=97/27%
- Negative Agreement Rate: 100/100×100%= 100%
- Overall rates of agreement: (100+107) / (3+100+0+107) ×100%=98/57%

Results of clinical assessment for Influenza A (negative percent agreement) NPA %100 and (positive percent agreement) PPA 100% and also for Influenza B (negative percent

agreement) NPA 100% and (positive percent agreement) PPA 90% obtained; results are in the following table.

Table 17: clinical assessment between simultaneous detection of nucleic acid Coronavirus Altona Diagnostics GmbH and ROJETechnologies.

Test		**RealStar®Influenza Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics GmbH)**				**Total**
		Positive (Influenza A)	Negative (Influenza A)	Positive (Influenza B)	Negative (Influenza B)	
Simultaneous detection Kit Viga SARS-COV-2 and Influenza A/B Molecular Diagnostic Kit	Positive (Influenza A)	30	0	-	-	30
	Negative (Influenza A)	0	100	-	-	100
	Positive (Influenza B)	-	-	27	0	27
	Negative (Influenza B)	-	-	3	100	103
Total		30	100	30	100	260

- Positive Agreement Rate (Influenza A): 30/30 ×100%＝100%
- Negative Agreement Rate (Influenza A): 100/100×100%= 100%
- Overall rates of agreement Rate (Influenza A): (100+30) / (0+100+0+30) ×100%= 100%
- Positive Agreement Rate (Influenza B): 27/30×100%=90%
- Negative Agreement Rate (Influenza B): 100/100×100%= 100%
- Overall rates of agreement Rate (Influenza B) :(100+27)/ (3+100+0+27) × 100 %=97/69 %

symbols

Tables 12: symbols on the Kit Label

symbols	meaning	symbols	meaning
	Date of manufacture		manufacturer
	Expiration Date		Temperature limitation
IVD	In Vitro Diagnostics	LOT	Lot number
		REF	Reference number

Troubleshooting

Here we try to cover as many problems as you may see in using this product; however, scientists in ROJE Technical Support Team are eager to answer all your questions. Do not hesitate to contact us for more information.

problems	Possible cause	suggestion
Negative control curves show an increase in Fluorescence intensity (a false positive happens)	• Sample contamination may occur.	• The performed test is invalid. Repeat the test, following the Kit instruction exactly.
Internal control or RNase P does not show any increase in fluorescence intensity curve.	• Execution of protocol is incorrect • Improper isolation of nucleic acid from a sample, resulting in loss of nucleic acid or Prescence of PCR inhibitor in a clinical sample • Lack of sufficient human cells in the sample to amplify in the PCR reaction	• Repeat the isolation of the sample, read the protocol carefully again and repeat the PCR.
Increasing the intensity of the Fluorescence signal does not show the state or shape of S.	• Low quality of extracted RNA samples. • Equipment failure, Real Time-PCR instrument.	• Repeat the test with extracted RNA. • Repeat the isolation of RNA with a valid Kit. • Diluted the isolated RNA to a ratio of1.10. • Repeat the test or contact the equipment supplier.

Ordering Information

category	Product name	Cat No.	Perps no.
Molecular diagnostic Kit	Viga SARS-CoV-2 and Influenza A/B Molecular Diagnostic Kit	MD983007	100 Preps
Related product	RNJia Virus kit	RN983072	100 Preps

Technical Assistance

ROJETechnologies guarantee your complete satisfaction. ROJE technical support team is composed of highly trained, experienced scientists; who can troubleshoot most problems you face. Our technical support team can offer expert advice which may help you select a suitable product.

Contact our technical support at any time by selecting one of these ways:

- Through our telephone and fax number; +982191070705.
- You can submit your question directly to ROJE Technical Support Team from our website (www.ROJETechnologies. com).
- Or send your questions to this email address, technicalsupport@rojetechnologies.com.

Factory address

NO. 2 Farvardin street- Fernan Street- Tehran- Shahr Qods- Iran- Postal Code: 37531146130- phone: +982191070705

ROJETECHNOLOGIES has been founded since 2014, and manufactures a wide range of molecular biology kits. We research, develop and create our products in order to make easier and more comfortable approaches to do research in molecular biology. Our target is offering high-quality affordable Molecular and diagnostic Kits and reagents, comparable of the world leaders, to research centers, laboratories, clinics, hospitals and diagnostic centers all over the world.

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