Stark SARS-CoV-2 & Influenza A/B Molecular Diagnostic Kit

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Contents

Description

The Stark SARS-CoV-2 & Influenza A/B Molecular Diagnostic Kit leverages a one-step RT-PCR reverse transcriptase reaction mechanism to accurately detect specific RNA sequences of the SARS-CoV-2 virus and both Influenza A and B viruses. Through a sophisticated process, a portion of the RNA sequence of each pathogen serves as a template for generating complementary DNA (cDNA), which then undergoes PCR amplification. The resulting PCR products are meticulously identified using fluorescently labeled oligonucleotide probes. Notably, this Kit identifies the N gene from SARS-CoV-2, the M2 gene from Influenza A, and the NS1 gene from Influenza B. It's important to highlight that this Kit exclusively targets these specific genes and does not identify other strains of coronaviruses or influenza viruses. The Primer/probe design for the N gene is strategically chosen to encompass a conserved region across all SARS-CoV-2 variants. The Kit utilizes fluorescence channels, including FAM, Yakima yellow, Texas Red, and Cy5, to precisely identify each targeted gene, enabling seamless compatibility with Real-Time PCR devices equipped with these channels.

Intended Use

The Stark SARS-CoV-2 & Influenza A/B Molecular Diagnostic Kit is designed for accurate detection of the N gene of SARS-CoV-2, the Matrix Protein gene of Influenza A, and the NS1 gene of Influenza B in individuals suspected of infection. Suitable samples include those obtained from the upper and lower respiratory tracts, such as nasopharyngeal swabs, throat swabs, nasal washes, nasal aspirations, sputum, and bronchoalveolar lavage. These samples may be collected from patients exhibiting symptoms indicative of COVID-19 or influenza or from healthcare personnel potentially exposed to these viruses. The Kit's comprehensive approach ensures accurate and reliable detection, aiding healthcare providers in timely diagnosis and appropriate management of patients.

Kit Content

Ingredients	100 Preps (REF: ST242003)
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

Upon receipt, store all reagents in a dark environment at -20 ±5ºC. Avoid frequent freeze-thaw cycles of the Kits. When stored according to the specified conditions, all reagents remain stable until the expiration date indicated on the Kit box.

Guarantee and Warranty

CARBON Technologies LLC stands behind the efficacy of all manufactured Kits and reagents. If you need assistance in choosing the right Kits for your needs, our technical support team is available to provide guidance. Should the products not meet your expectations due to reasons other than misuse, please do not hesitate to contact our technical support team. In the rare event of issues arising from the manufacturing process, CARBON Technologies LLC will promptly replace the Kit.

Warning and Precautions

- Ensure that only personnel who are specially trained and instructed in handling RT-PCR diagnostic cases utilize this product.
- Regular maintenance and repair of the Real-Time PCR instrument are necessary.
- Clean tables and equipment periodically to maintain hygiene standards.
- Utilize sterile filter pipette tips that are free from RNase and DNase.
- Always treat samples as infectious and handle them with care according to laboratory safety protocols. Wear powder-free latex gloves when handling Kit materials and components.
- Establish separate workstations for sample preparation and reaction setup. Each workstation should be equipped with dedicated tools and equipment, and airflow should be directed from pre-PCR to post-PCR areas.
- Exercise caution when working with samples containing high virus titers and positive controls to prevent laboratory contamination.
- Change gloves after handling samples or positive controls, and keep them segregated from other reaction materials.
- Prevent contamination of workstation materials and equipment with DNA/RNA and nucleases.
- Ensure that the RNA isolation method is compatible with the RT-PCR reverse transcriptase technique to maintain the quality of isolated RNA.
- To prevent false-positive results due to viral RNA contamination during isolation, use a negative control (water instead of sample) during RNA extraction and PCR testing. Optionally, include a negative control sample (water instead of sample) in each PCR reaction.
- Monitor the expiration date of the Kit and avoid repeated freezing and thawing of its components. Store Kit components away from light.
- Dispose samples following laboratory waste disposal safety regulations.

Quality Control

In adherence to standards set by the Clinical and Laboratory Standards Institute and WHO, the STARK SARS-COV-2 Molecular Diagnostic Kit undergoes rigorous testing across multiple experiments on a lot-to-lot basis to guarantee consistent product quality. Detailed results of these experiments are accessible online by referencing the REF and Lot numbers at www.carbontechnoloesco.com.

Materials Required (but Not Provided)

Here are the materials required for the STARK SARS-COV-2 Molecular Diagnostic Kit, which are not provided:

- 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 pipettes and pipette tips (10µl, 100µl, and 1000µl of filter pipette tips).
- Surface sanitizing solution.
- 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 Instruments

The STARK SARS-COV-2 Molecular Diagnostic Kit is compatible with the following Real-Time PCR 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

Please note that some PCR devices may require calibration with the desired colors before performing multiplex-PCR reactions. For further details about specific Real-Time PCR devices, refer to the device manual template.

Applications

The Stark SARS-CoV-2 & Influenza A/B Molecular Diagnostic Kit utilizes a one-step RT-PCR reverse transcriptase reaction to detect specific RNA sequences from pathogens. This process involves generating complementary DNA (cDNA) from the RNA template of the pathogen, followed by PCR amplification. The resulting PCR products are then identified using oligonucleotide probes labeled with fluorescent colors. This Kit is designed to detect the N gene from SARS-COV-2 (Coronavirus), the M2 gene from Influenza A, and the NS1 gene from Influenza B virus. Other Coronaviruses and strains of the Influenza virus are not detected by this Kit.

The primer/probe sets for the N gene of COVID-19, the M gene of Influenza A, and the NS1 gene of Influenza B are designed to target conserved regions among their respective pathogens. These genes are detected in specific fluorescence channels: the N gene in the orange channel, the M gene in the green channel, and the NS1 gene in the yellow channel. The internal control, RNase P gene, is detected in the Cy5 channel, serving as a control to isolate RNA and check for RT-PCR reaction inhibition. This Kit is compatible with Real-Time PCR devices capable of identifying four fluorescence channels (green, yellow, orange, and red).

Recommended Starting Material

Sample Collection

All samples must be collected to avoid contamination during sampling, storage, and transportation. Samples should be treated as potentially infectious, following biosafety guidelines. Synthetic-tipped swabs with aluminum or plastic shafts are recommended for collection, while cotton swabs with wooden shafts should be avoided. After sampling, swabs should be stored immediately in a virus transport medium.

Storage and Delivery of Specimens

Samples should be tested within 24 hours if stored at 4ºC. For longer storage, samples can be stored at -70ºC or below. If -70ºC storage is unavailable, specimens can be stored at -20ºC for up to ten days, and nucleic acid can be stored at -20±5ºC for 15 days. Avoid multiple freeze-thaw cycles to maintain sample integrity.

Specimen Isolation

For viral nucleic acid isolation, use a Kit which is approved by the Ministry of Health.

Pathogenicity

Coronaviruses belong to the Coronaviridae family, characterized by positive single-stranded RNA genomes. Strains such as HCOV-229E, HCOV-NL63, HCOV-OC43, MERS-CoV, and HCOV-HKU1 are known to cause various respiratory illnesses in humans, including the common cold, upper respiratory tract infections, bronchiolitis, and pneumonia. SARS-CoV-2, identified as a beta coronavirus in December 2019 in Wuhan, China, is the causative agent of COVID-19. Fever, cough, and respiratory issues are among the primary symptoms, often progressing to pneumonia and severe respiratory syndrome (SARS). The virus primarily spreads through close contact with respiratory droplets. Influenza is an acute infectious disease caused by Influenza A and B viruses, with Influenza C viruses causing fewer cases. The genome of influenza viruses comprises segmented RNA strands encased in a protein capsid with a negative head. These viruses are ubiquitous globally. The spread of influenza A is primarily attributed to antigenic drift of hemagglutinin and neuraminidase molecules. While Influenza B and C viruses are predominantly human pathogens, Influenza A viruses have a broader host range, infecting various warm-blooded animals.

Workstation Preparation

Before commencing any procedures, it is essential to prepare the workstation diligently. Thorough cleaning and sanitization of all work surfaces, pipettes, centrifuges, and other equipment are imperative. To minimize the risk of nucleic acid contamination, utilize sanitizers such as 70% Ethanol or 10% Sodium Hypochlorite. This ensures a clean and safe environment conducive to accurate testing procedures.

Protocol

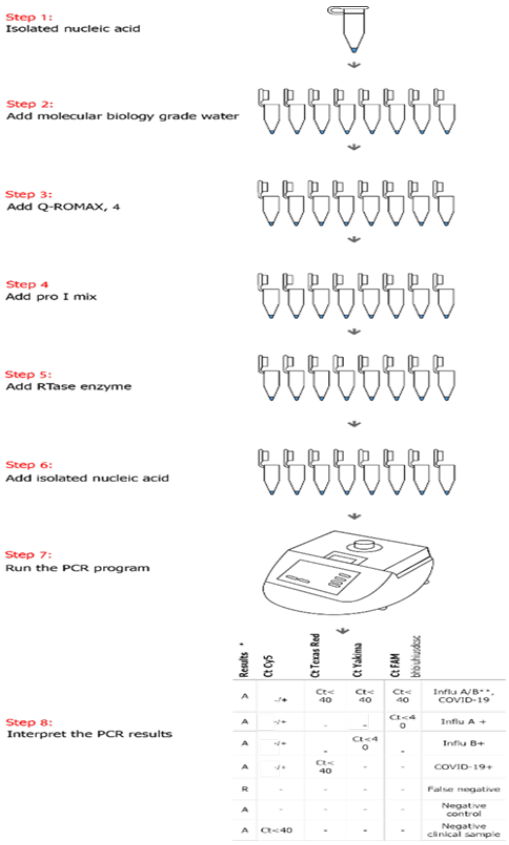

Figure 1: Preparation of reagents, the addition of isolated DNA, PCR run, and interpretation of results

Retrieve each component from the diagnostic Kit and set them aside at room temperature. Let the reagents equilibrate to room temperature before proceeding, and then briefly vortex each to ensure proper mixing for later use. Ensure that the volume of the isolated sample used in this test is 10µl. Refer to table 1 to prepare the reaction components accordingly and conduct Real-Time PCR as outlined in table 2.

Table 1: 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	3min	95ºC	1
Denaturation	10s	95ºC	45 cycles
Annealing and extension of nucleic acid and measurement of fluorescence in green, yellow, and orange 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 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 green, yellow and orange channels indicate Influenza A, Influenza B, and COVID-19, respectively. 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.

- A positive clinical specimen should have Ct≤40 for each of the three genes or positive genes.
- 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<40	Influenza A/B and positive COVID19
Unacceptable	Negative	Negative	Negative	Negative	False negative
Acceptable	Negative	Negative	Negative	Ct< 40	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 Limitations

The efficacy of this test is contingent upon the collection, handling, and storage of samples. Specifically designed for diagnosing targeted viruses in swab samples and respiratory sputum, this Kit performs optimally when utilized on samples obtained from individuals displaying symptoms of COVID-19 or influenza. Samples may be self-collected or obtained by healthcare personnel. A negative test result does not definitively rule out the presence of SARS-CoV-2, Influenza A, or B viruses, as various factors such as sample collection technique, user error, mixing methodology, or low viral titers can potentially impact the accuracy of the test. Additionally, the presence of PCR inhibitors may lead to false-negative results. Moreover, sequence variations in the target regions of unknown viruses may also contribute to false-negative results or reduced Kit sensitivity. Therefore, the interpretation of results should be based on clinical assessments and complementary diagnostic tests.

Performance Evaluation

- Target Gene Selection and Primer/Probe Design

Sequence data for Influenza A, Influenza B, and SARS-CoV-2 were retrieved from NCBI archives. Utilizing bioinformatics tools such as Mega ten and Claustral, alignment analysis was conducted to identify the most conserved regions within each target gene. Primers and probes were designed using Beacon Designer and AlleleID 7.5 software based on the selected conserved regions. Subsequently, the specificity and characteristics of the

designed primers and probes were evaluated using Oligo7, Gene runner, and NCBI blast.

- **Preparation of Standard Samples**

Standard RNA samples were prepared with concentrations of 200,000 copies/mL for SARS-CoV-2 and 15,000 copies/mL for Influenza A and Influenza B, utilizing AMPLIRUN® INFLUENZA A H1 RNA CONTROL and AMPLIRUN® INFLUENZA B RNA CONTROL controls. From these samples, dilutions of 1/10, 1/100, and 1/1000 were prepared by diluting 100µl of the initial sample with 900µl of water. The average of six repetitions was performed using the Novel Coronavirus (2019-nCOV) Nucleic Acid Diagnostic Kit (Sansure Biotech Inc) for diagnosing SARS-CoV-2, and the RealStar®Influenza Screen & Type RT-PCR Kit 4.0 (Altona Diagnostics GmbH) for diagnosing Influenza A and B. Results are presented 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

The diluted specimens were subsequently tested for the simultaneous detection of nucleic acid, targeting both Stark SARS_COV_2 and Influenza A/B. The results are presented 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	Prepared concentration for testing [copies/ml]	Target genes	test for simultaneous diagnosis using a nucleic acid detection Kit (Stark Influenza A/Influenza B/Coronavirus 2

detection Kit (Stark Influenza A/Influenza B/Coronavirus 2 Real-Time RT-PCR Kit)			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 conducted to establish the lowest detectable concentration of RNA from Influenza A/B and Coronavirus 2, where approximately 95% of all true positive replicates yield positive test results.

To determine the LoD, dilutions containing 200 copies/ml of novel Coronavirus and 150 copies/ml of Influenza A and B were prepared. These dilutions were further diluted to concentrations of 200, 100, and 50 copies/ml for Coronavirus, and 150, 75, and 35 copies/ml for Influenza A and B, respectively. Each dilution was then tested in eight replicates. The obtained results indicate that the lowest limit of detection for the Stark SARS-COV-2 Molecular Diagnostic Kit is 100 copies/ml for Coronavirus and 200 copies/ml for Influenza A/B when using the nucleic acid simultaneous detection Kit (Stark SARS-CoV-2 & Influenza A/B Molecular Diagnostic Kit Real-Time RT-PCR).

Table 7: The lowest detectable concentration of Influenza A/ Influenza B and Coronavirus 2 using Stark 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	37.824689	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	39.423	40.523	Undeter	36.634	Undetermi	Undetermi	35.319	37.301	Undetermi

	1		mined	2	ned	ned	48325	05653	ned
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 each concentration	100%	62.5%	25%	100%	50%	-	100%	87.5%	-

- **Analytical Sensitivity**

The primer/probe sets utilized in the simultaneous detection of nucleic acid (Stark SARS-COV-2 & Influenza A/B Molecular Diagnostic Kit) were subjected to in-silico analysis for SARS-COV-2 and Influenza A/B on the NCBI platform. Alignment analysis of primer/probe sequences targeting the N gene of COVID-19, M gene of Influenza A, and NS1 gene of Influenza B revealed a 100% overlap with the sequences of each respective virus. The alignment results for each gene are detailed 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_4039 32	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	N gene	MW349104.1	100	100	100

coronavirus 2 isolate SARS-CoV-2/human/USA/MN-MDH-2049/2020					
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	M1	MT644554.	100	100	100
Carolina/A02478981/2020(H1N1)		1			
matrix protein 1 (M1)					
Influenza A virus	M1	MT644542.	100	100	100
(A/swine/Iowa/A02478968/2020(H1		1			
N2) matrix protein 2 (M2)					
Influenza A virus	M1	MT639878.	100	100	100
(A/Texas/9804/2019(H3N2))		1			
segment 7 matrix protein 2 (M2)					
Influenza A virus (A/South	M1	MT639798.	100	100	100
Korea/9793/2019(H3N2)) segment 7		1			
matrix protein 2 (M2)					
Influenza A virus (A/South	M1	MT639790.	100	100	100
Korea/9792/2019(H3N2)) segment 7		1			
matrix protein 2 (M2)					
Influenza A virus	M1	MT638933.	100	100	100
(A/Ohio/9270/2019(H3N2)) segment		1			
7 matrix protein 2 (M2)					
Influenza A virus (A/Mallard	M1	MT624468.	100	100	100
duck/Alberta/471/2019(H3N8))		1			
segment 7 matrix protein 2 (M2)					
Influenza A virus	M1	MN948660.	100	100	100
(A/Iowa/59/2019(H3N2)) segment 7		1			
matrix protein 2 (M2)					
Influenza A virus	M1	MN948318.	100	100	100
(A/Oklahoma/24/2019(H1N1))		1			
segment 7 matrix protein 2 (M2)					
Influenza A virus	M1	MN948118.	100	100	100
(A/California/196/2019(H1N1))		1			
segment 7 matrix protein 2 (M2)					

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

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	NS1	MT315021.1	100	100	100

protein 1 (NS1) genes					
Influenza B virus (B/Iowa/37/2019) protein 1 (NS1) genes	NS1	MT314997.1	100	100	100

- Clinical Sensitivity

To assess clinical sensitivity, both negative (pharyngeal and nasal swabs) and positive specimens were prepared and diluted accordingly. The specimens were then extracted, and the test was conducted using the simultaneous detection of nucleic acid Kit (Stark SARS-COV-2 & Influenza A/B Molecular Diagnostic Kit), with each test repeated three times. The results of these repetitions are outlined in the following table:

Table 11: Result of clinical sensitivity test for simultaneous nucleic acid detection (Stark 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)

In silico analysis was conducted for the simultaneous detection of nucleic acid (Stark SARS-COV-2 & Influenza A/B Molecular Diagnostic Kit) to assess cross-reactivity with other respiratory pathogens. Primer/probe sequences for the M2, NS1, and N genes of Influenza A/B and Coronavirus were aligned with sequences in the NCBI nr/nt database using BLASTN 2.10.0+ software. The analysis confirmed the detection of the M2, NS1, and N genes of Influenza A, B, and Coronavirus, respectively, with no observed cross-reactivity with other respiratory pathogens. The results of this analysis are detailed 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	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/SC9	DQ437612.1	30	36	71

	428/2018				
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/ACR I-051/2016	LR739069.1	46	72	60
Mycobacterium tuberculosis	C9_S	AF269311.1	46	76	52
Human Metapneumo virus (hMPV)	MY/U2311/201 3	KU320918.1	45	67	62
Parainfluenza virus 1-4	HPIV3/Seattle/ USA/7J10/201 1	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_01837587 6.1	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 Metapneumo virus (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/Kan a-Ebi6/2019/G8-2/PL-CP	LC549660.1	55	42	58
Respiratory syncytial virus	GH300327/US A/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	2245STDY6178 854	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_01837515 4.1	48	56	49
Candida albicans	TIMM 1768	CP032019.1	75	43	58
Pseudomona s aeruginosa	paerg000	LR130528.1	58	63	67
Staphylococc us epidermis	SESURV_p4_1 553	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	Strain	GenBank Acc#	% Homology Test Probe	% Homology Test RP	% Homology Test FP

ID)					
Paramyxoviri dae	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	YK41	LN849050.1	66	65	73

pneumoniae					
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)

The clinical specificity of nucleic acid detection for respiratory pathogens such as Adenovirus, Legionella Pneumophila, Neoformans, Chlamydia Pneumoniae, Streptococcus Pneumonia, Respiratory Syncytial Virus, Mycoplasma Pneumoniae, Streptococcus Pyogenes, and Mycobacterium Tuberculosis was evaluated using negative swab specimens diluted to a specific concentration. These samples were isolated and tested using the simultaneous detection of nucleic acid (Stark SARS-COV-2 & Influenza A/B Molecular Diagnostic Kit). No cross-reactivity was observed for the respiratory pathogens listed in the table below:

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

Virus / Bacteria / Parasite	Source / Sample type	Concentration	Ct Value (ORF1ab gene/N gene)
Adenovirus	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Legionella pneumophila	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Cryptococcus neoformans	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Chlamydia pneumonia	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Streptococcus pneumoniae	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Respiratory Syncytial Virus	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-

Mycoplasma pneumoniae	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Streptococcus pyogenes	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
Mycobacterium tuberculosis	AmpliRun®DNA/RNA Vircell	104 copies/ml	-/-
10 Pooled human genomes	Clinical sample	10 ng/µl	-/-

- Accuracy Assessment

Accuracy assessment comprises both In Vitro Intra-assay and Inter-assay evaluations.

Intra-assay

Intra-assay evaluates the precision and capability of the method to determine the concentration of similar repeats within one Real-Time PCR cycle, represented as standard deviation (SD) for different threshold cycle (Ct) values. Three repetitions of each concentration of the control sample were examined in one reaction, and coefficient of variation (CV) values were calculated for the threshold cycle values. For the Coronavirus (N gene), the maximum coefficient of variation is 0.63, with a minimum of 0.20. For Influenza A (M gene), the maximum coefficient of variation is 2.5, with a minimum of 0.36. For the Influenza B virus (NS1 gene), the maximum coefficient of variation is 1.8, with a minimum of 0.73. All acceptable results must have a CV of less than 5%.

Inter-assay

Inter-assay evaluates the results from different runs in Real-Time PCR or other laboratories, typically expressed as SD or CV for different Ct values related to the number of copies or different concentrations of a sample. Real-Time PCR reactions, with at least five repeats of each concentration of the control sample, were tested on three other days. For the Coronavirus (N gene), the maximum coefficient of variation is 4.4, with a minimum of 0.8. For Influenza A virus (M gene), the maximum coefficient of variation is 3.3, with a minimum of 1.75. For Influenza B (NS1 gene), the maximum coefficient of variation is 4.08, with a minimum of 0.06. All acceptable results must have a CV of less than 10%.

- Clinical Evaluation

The clinical performance of the simultaneous detection of nucleic acid (Stark SARS-COV-2 Molecular Diagnostic Kit) was assessed using 100 negative samples, 110 positive COVID-19 samples, 30 positive Influenza A samples, and 30 positive Influenza B samples obtained from throat and nasal swabs collected from patients suspected of having COVID-19.

To compare and validate the simultaneous detection of nucleic acid (Stark SARS-COV-2 & Influenza A/B Molecular Diagnostic Kit), two other Kits, the Novel Coronavirus (2019-nCoV) Nucleic Acid Coronavirus Diagnostic Kit (PCR Fluorescence Probing) from Sansure Biotech Inc, and the RealStar®Influenza Screen & Type RT-PCR Kit 4.0 from Altona Diagnostics GmbH, which were licensed for emergency use in the laboratory by the US Food and Drug Administration, were also evaluated.

Results of the clinical assessment for COVID-19 showed a negative percent agreement (NPA) of 100% and a positive percent agreement (PPA) of 97.27%, as shown in the table below.

Table 16: Clinical assessment between simultaneous detection of nucleic acid Novel Coronavirus, Sansure Biotech, and CARBON Technologies LLC.

Test	Novel Coronavirus Nucleic Acid Diagnostic Kit (PCR Fluorescence Probing) (Sansure Biotech Inc)		Total
	Positive	Negative	

Simultaneous detection Kit (Stark 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 the clinical assessment for Influenza A revealed a negative percent agreement (NPA) of 100% and a positive percent agreement (PPA) of 100%. Similarly, for Influenza B, the negative percent agreement (NPA) was 100%, and the positive percent agreement (PPA) was 90%. These findings are detailed in the table below.

Table 17: Clinical assessment between simultaneous detection of nucleic acid Coronavirus Altona Diagnostics GmbH and CARBON Technologies LLC.

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 Stark SARS-COV-2 & 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

	Manufacturer
	Date of manufacture
	Use-by date
	Contains sufficient for <n> tests
	Consult instructions for use
	Biological risks
	Temperature limit
	CE Marking
	EU Representative
	In Vitro diagnostic medical device
	Catalogue Number
	Lot Number

Troubleshooting

We strive to address potential issues that may arise in using this product. However, our team at CARBON Technologies Technical Support is dedicated to addressing any questions or concerns you may have. Please feel free to reach out to us for further information or assistance.

Problem	Possible Causes	Suggestion
Negative control curves show an increase in Fluorescence intensity (a false positive happens).	Sample contamination might have occurred.	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.

Technical assistance

For technical assistance, CARBON Technologies LLC ensures your complete satisfaction. Our technical support team comprises highly trained and experienced scientists adept at troubleshooting most problems you may encounter. They can offer expert advice to help you select the most suitable product for your needs.

You can contact our technical support team anytime through the following methods:

- Phone: +96897058350
- Directly submit your questions to the CARBON Technologies technical support team through our website: www.carbontechnologiesco.com
- Email your questions to: technicalsupport@carbontechnologiesco.com

Rest assured, our team is dedicated to providing prompt and effective assistance to address any inquiries or issues you may have. We are committed to ensuring your satisfaction and success in using our products.

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