FLU SC2 RT-PCR

Qualitative assay for use on RT-PCR System

In vitro Diagnostic Use

Instructions for Use

Intended use

FLU SC2 RT-PCR is a real-time RT-PCR test intended for the qualitative detection of RNA from influenza A, influenza B, and SARS-CoV-2 in nasopharyngeal swabs collected in commercially available Viral Transport Media (VTM) from individuals suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider. Symptoms of respiratory viral infection due to SARS-CoV-2 and influenza can be similar.

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.

Results are for the presumptive detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA in patient specimens and is not intended to detect influenza C. RNA from influenza A, influenza B, and/or SARS-CoV-2 viruses is generally detectable in nasopharyngeal swabs during the acute phase of infection. Positive results are indicative of active infection but do not rule out bacterial infection or co-infection with other viruses; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all SARS-CoV-2 test results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2, influenza A, and/or influenza B infection and should not be used as the sole basis for diagnosis, treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and/or epidemiological information. The agent detected may not be the definite cause of disease.

Explanation of the test

The FLU SC2 RT-PCR Assay is a molecular in vitro diagnostic test that aids in the detection and differentiation of RNA from influenza A virus, influenza B virus or/and SARS-CoV-2 and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes, and control material used in RT-PCR for the in vitro qualitative detection and differentiation of RNA of influenza A virus, influenza B virus or SARS-CoV-2 in upper and lower respiratory specimens such as nasopharyngeal (NP) swabs. The RNA Internal Control, used to monitor the entire sample preparation and PCR amplification process, is introduced into each specimen during sample processing. In addition, the test utilizes external controls (positive control and a negative control).

Principles of the procedure

The FLU SC2 RT-PCR test includes four primer-probe sets corresponding to those used in the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Real-Time RT-PCR diagnostic panel (CDC Catalog # Flu-SC2-EUA and FDA submission number EUA201781).

FLU SC2 RT-PCR protocol includes the extraction of RNA by commercially available kits with recommended proper precautions, which renders samples as non-infectious. Nucleic acid from patient samples are extracted and detection of influenza A, influenza B and SARS-CoV-2 is carried out by the extracted RNA from the nasopharyngeal samples.

The three primer and probe sets aid to detect one region each for influenza A, influenza B, and SARS-CoV-2, and one primer and probe set to detect human RNase P in a clinical sample. The primer-probe set specific to detect RNase P, is included to serve as an internal reference to monitor nucleic acid exposure.

FLU SC2 RT-PCR uses fluorescent probes and specific primers to detect specific regions of the Influenza A {Matrix, (M1) gene}, Influenza B {Nonstructural 2 (NS2) gene}, and Covid-19 (N gene). RNA is reverse transcribed into cDNA by reverse transcriptase. Primer specific regions of cDNA are then amplified by PCR. During the extension phase of the PCR cycle, the 5′ nuclease activity of Taq polymerase degrades the probe; the reporter dye will be separated from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. External controls (positive and negative) are processed in the same way with each FLU SC2 RT-PCR run.

The materials provided for FLU SC2 RT-PCR can be found in the below Table.

Reagents for FLU SC2 RT-PCR

Component	Vials Per Kit	State	Application
Flu SC2 Master Mix	1	Liquid	Buffering components and enzymes
Flu SC2 Combined Primer-Probe Mix	1	Liquid	Includes targets for InfA, InfB, SC2, and RNase P
Combo PC (Positive Control)	1	Liquid	Yields a positive result for InfA, InfB, SC2 and RNase P
Nuclease Free water (No Template Control)	1	Liquid	Used as negative template control

Equipment and Consumables Required (But Not Provided)

- 1.5 mL polypropylene microcentrifuge tubes (DNase/ RNase free)
- Disposable powder-free gloves and surgical gowns
- -70°C and -20°C freezer(s)
- 4°C refrigerator
- 2 x 96-well cold blocks (-20°C)
- Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- Real-Time PCR Instrument
- RNA Extraction kit
- Benchtop microcentrifuge
- Vortex mixer

Good laboratory practice

- Do not pipette by mouth.
- Do not eat, drink, or smoke in designated work areas.
- Wear laboratory gloves, laboratory coats, and eye protection when handling samples and reagents. Gloves must be changed between handling samples and FLU SC2 RT-PCR to prevent contamination. Avoid contaminating gloves when handling samples and controls.
- Wash hands thoroughly after handling samples and kit reagents, and after removing the gloves.
- Thoroughly clean and disinfect all laboratory work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in distilled or deionized water (dilute household bleach 1:10). Follow by wiping the surface with 70% ethanol.
- If spills occur on the instrument, follow the instructions in the RT-PCR Systems – User Assistance and/or User Guide to properly clean and decontaminate the surface of instrument(s).

Storage and Shelf life

All components of FLU SC2 RT-PCR kit must be stored at -20°C with protection from light. Reagents are stable for 12 months when stored at the recommended conditions. Repeated freezing and thawing may lead to inaccurate results. Do not use the kit after expiry date or if the pack is damaged. We recommend disposing unused or expired kit according to federal, country and local bio-safety regulations.

Sample Collection and Storage

Collect fresh nasopharyngeal swab specimen in VTM from individuals suspected of respiratory infection. Specimen collection should avoid possible contamination during collection, storage, and transportation according to regulatory guidelines by CDC or FDA. Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV).[2,3] The specimen may be tested immediately after collection or it may be stored at 2-8°C for up to 72 hours before testing. The specimen should be shipped in low temperature conditions using dry ice or ice pack.

Sample processing

RNA extraction is performed according to the commercially available kits. Extracted RNA will be used as template for the RT-PCR reaction.

Analytical protocol

1. In the reagent set-up room, clean the biosafety cabinet, place Flu SC2 Master Mix and Primer/Probes on ice or cold-block.
2. RNA of patient samples on ice or cold block until the moment of amplification.
3. Centrifuge all the components for 5-10 seconds to collect contents at the bottom of the tube, and then the tubes are placed in a cold rack.
4. Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the negative and positive controls and specimens for pipetting error.
5. Prepare the reaction mix by combining the Flu SC2 Master Mix and Flu SC2 Primers and Probe set as described below:

Component	Volume
Flu SC2 Master Mix	11.5 µL
Flu SC2 Primers and Probe set	3.5 µL

6. Add 5.0 µL of the RNA samples and controls to the appropriate wells/tubes.
7. Centrifuge for 5-10 seconds and set up reaction tubes or plates in a cooler rack. The samples are now ready for thermal cycling.

Selection of Fluorescence channel

Target	Gene	Dye	Color
SC2	N Gene	FAM	Green
INF-A	M2 Gene	TexRd	Orange
INF-B	NS2 Gene	Hex	Yellow
RNase P	RNase P	Cy5	Far-Red

Thermal Cycling

Step	Cycles	Temperature	Time
cDNA Synthesis	1	50 ^{0}C	15 min
Initial Denaturation	1	95 ^{0}C	2 min
PCR cycling	45	95 ^{0}C	5 Sec
		60 ^{0}C	30 Sec

Control Materials to be Used with Flu SC2 Multiplex RT-PCR:

- A negative control (Nuclease free water) is needed to confirm there has been no sample contamination on the assay run and is used on every assay run.
- A positive control (InfA, Inf B, Covid-19 and RNase P) is needed to verify that the assay run is performing as intended and is used on every assay run.
- An Internal control (RNase P) targeting human RNase P gene is needed to verify that nucleic acid is present in every sample and is used for every sample processed to ensure that samples resulting as negative contain nucleic acid for testing.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory standard quality control procedures. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1200.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples and with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- HSC extraction control must proceed through nucleic acid isolation with each batch of specimens to be tested.
- Always include a negative control (NTC), and the appropriate positive control (i.e., Combo Flu SC2 PC) in each amplification and detection run.

INTERPRETATION OF RESULTS

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the test results cannot be interpreted.

FLU SC2 RT-PCR – Positive, Negative and Internal Controls

The expected results generated from each control and acceptance criteria are as follows:

- Negative (no template) Control – Negative for all targets detected (Ct Not Detected or Ct>38)
- Positive Control – Positive for all targets detected (Ct < 38)
- Internal Control –Positive for RNase P (Ct <38)
- If controls do not perform as described above, the run is considered invalid and the specimen must be reanalyzed, and the test steps should be repeated from the initial step. Expected performance of controls included in the FLU SC2 RT-PCR Diagnostic Panel.

Control Type	External Control Name	Used to Monitor	InfA	InfB	SC2	RNase P	Expected Ct Values
Positive	Combo PC	RT-PCR reagent integrity including primers and probes for all targets	+	+	+	+	< 38.00 Ct for InfA, InfB, SC2 and RNase P
Extraction	RNase P (Human specific control)	Extraction reagent integrity, contamination	-	-	-	+	< 38.00 Ct
Negative	NTC	Contamination of reagents or environmental contamination	-	-	-	-	Ct>38 or Not detected

Examination and Interpretation of Patient Specimen Results

Assessment of clinical specimen test results should be performed after the positive, negative (no template) and internal controls have been examined and determined to be valid and acceptable. If the RNAase P control does not produce a positive result for human clinical specimens, interpret as follows:

1. If Inf A/ Inf B and Covid-19 (N) gene is positive even in the absence of RNAase P, the result should be considered valid. It is possible that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RNAase P signal does not preclude the presence of INF A/ INF B and Covid-19 virus RNA in a clinical specimen.

2. If Inf A/ Inf B/ Covid-19 (N) and RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible. If the controls are not valid, patient results cannot be interpreted.

InfA Result	InfB Result	SC2 Result	RNase P Result	Interpretation	Report	Actions (Specimen from clinical sites)
+	-	-	±	influenza A RNA detected	Positive for influenza A	Report results to sender
-	+	-	±	influenza B RNA detected	Positive for influenza B	Report results to sender
-	-	+	±	SC2 RNA detected	Positive for COVID-19	Report results to sender
-	+	+	±	influenza B and SC2 RNA detected	Positive for influenza B and COVID-19	Report results to sender
+	+	-	±	influenza A and influenza B RNA detected	Positive for influenza A and Influenza B	Report results to sender
+	-	+	±	influenza A and SC2 RNA detected	Positive for Influenza A and COVID-19	Report results to sender
+	+	+	±	influenza A, influenza B and SC2 RNA detected	Positive for influenza A, influenza B and COVID19	Report results to sender
-	-	-	+	Not Detected	Negative	Report results to sender. Consider testing for other respiratory viruses
-	-	-	-	Invalid result	Invalid	Consider repeat of extraction and/or rRTPCR or collecting a new specimen

Cut-off for all targets:

Ct value	Result
≤ 38	Detected
> 38 or N/A	Not detected

If there is no typical S-shape amplification curve or Ct > 38 or No Ct detected for Inf A, Inf B, Covid-19 (N) or RNAase P, it indicates that the specimen concentration is too low, or there are interfering substances that inhibit the reaction. If upon retest, the result is invalid again, another fresh sample should be collected and tested.

Procedural limitations

- Reliable results depend on proper sample collection, storage and handling procedures.
- Detection of SARS-CoV-2 and Influenza A/B RNA may be affected by sample collection methods, patient factors (e.g., presence of symptoms), and/or stage of infection.
- As with any molecular test, mutations within the target regions of FLU SC2 RT-PCR could affect primer and/or probe binding resulting in failure to detect the presence of virus.
- Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next; users perform method correlation studies in their laboratory to qualify technology differences. One hundred percent agreement between the results should not be expected due to aforementioned differences between technologies. Users should follow their own specific policies/procedures.
- False negative or invalid results may occur due to interference. The Internal Control is included in FLU SC2 RT-PCR to help identify the specimens containing substances that may interfere with nucleic acid isolation and PCR amplification.
- Results (positive and negative) for influenza should be interpreted with caution. If an influenza result is inconsistent with clinical presentation and/or other clinical and epidemiological information, FDA-cleared Influenza NAATs are available for confirmation if clinically indicated.

PERFORMANCE EVALUATION

Limit of Detection (LoD) - Analytical Sensitivity:

The LoD study demonstrated the lowest concentration of Influenza A, Influenza B and SARS-CoV-2 (genome copies/µL) that can be detected by the FLU SC2 RT-PCR kit as per the analytical protocol. It was performed using Influenza A H1 RNA (Vircell, Cat no. MBC028-R), Influenza B RNA (Vircell, Cat no. MBC030-R) and heat inactivated SARS-CoV-2 (BEI Resources, Cat no. NR-52286) with the defined number of gene copies on Qiagen Rotor-Gene Q5Plex HRM instrument. Five different serial dilutions (1000 copies/µL, 100 copies/µL 50 copies/µL, 25 copies/ µL and 12.5 copies/µL) of the RNA of Influenza A, Influenza B and SARS-CoV-2 were used in triplicate. The established LoD of the FLU SC2 RT-PCR Kit assay was 25 copies/ µL for InfA, 12.5 copies/µL for InfB and 12.5 copies/µL of SC2 and the results of the LoD confirmatory study are summarized below.

FLU SC2 RT-PCR – Qiagen Rotor-Gene Q5Plex				
Number of copies	**Number of replicate**	**InfA % Positive**	**InfB % Positive**	**SC2 % Positive**
1000 copies/ µL	03	100%	100%	100%
100 copies/ µL	03	100%	100%	100%
50 copies/ µL	03	100%	100%	100%
25 copies/ µL	03	100%	100%	100%
12.5 copies/ µL	20	95%	100%	100%

Inclusivity (Analytical Sensitivity)

CDC has granted developers the right of reference to the CDC Flu SC2 Multiplex Assay. The right of reference allows the developers seeking EUA for a multi-analyte respiratory panel that includes Flu SC2, to use the performance data already submitted to FDA as part of the CDC EUA request for the Flu SC2 Multiplex Assay (FDA submission number EUA201781). Biogenex claims the right of reference to the CDC EUA performance data (LoD, Inclusivity (analytical sensitivity), Cross-reactivity, Co-Infection Sensitivity) for the FLU SC2 RT-PCR kit.

The FLU SC2 RT-PCR kit utilizes identical oligonucleotide sequences for the Influenza A, Influenza B and SARS-CoV-2 target genes to those used in the CDC recommended 2020-CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Real-Time RT-PCR Primers and Probes Diagnostic Panel. The inclusivity and cross-reactivity of the CDC EUA Flu SC2 Multiplex Assay has been evaluated previously and therefore, additional evaluation was not necessary.

Analytical specificity (cross-reactivity and microbial interference)

The cross-reactivity of the CDC EUA assay has been evaluated previously and therefore, additional wet lab evaluation was not necessary. CDC has provided a right of reference to their cross-reactivity study data (FDA Submission number EUA201781), which is available at **https://www.fda.gov/media/139743/download.**

To further assess for potential cross-reactivity of the FLU SC2 RT-PCR test, an in silico analysis of the Influenza A, Influenza B and SARS-CoV-2 sequences was performed using BLASTN 2.10.1 against partial or complete genomes of other common respiratory viral and bacterial pathogens listed in the below table. None of the pathogen sequences displayed greater than 80% homology with any of the Influenza A, Influenza B and SARS-CoV-2 primers/probes.

Additionally, to bridge use of the assay with the Quant Studio 5 (HRM Software v2.0) and Biorad CFX96 instrument, LoD was determined using the instrument with the FLU SC2 RT-PCR kit as per the analytical protocol. It was performed using Influenza A H1 RNA (Vircell, Cat no. MBC028-R), Influenza B RNA (Vircell, Cat no. MBC030-R) and heat inactivated SARS-CoV-2 (BEI Resources, Cat no. NR-52286). The different serial dilutions (1000 copies/ µL, 100 copies/ µL, 50 copies/µL, 25 copies/ µL and 12.5 copies/µL) of the quantified RNA Influenza A, Influenza B and quantified heat inactivated SARS-CoV-2 were used. The established LoD of the FLU SC2 RT-PCR assay on Quant Studio 5 instrument and BioRadCFX96 was 12.5 copies/µL for InfB and SC2 and 25 copies/ul for InfA. The results of the LoD confirmatory study are summarized below.

LoD study of FLU SC2 RT-PCR using Quant Studio 5

FLU SC2 RT-PCR – Quant Studio 5				
Number of copies	**Number of replicate**	**InfA % Positive**	**InfB % Positive**	**SC2 % Positive**
1000 copies/ µL	03	100%	100%	100%
100 copies/ µL	03	100%	100%	100%
50 copies/ µL	03	100%	100%	100%
25 copies/ µL	03	100%	100%	100%
12.5 copies/ µL	20	95%	100%	100%

LoD study of FLU SC2 RT-PCR using Biorad CFX96

FLU SC2 RT-PCR – Biorad CFX96				
Number of copies	**Number of replicate**	**InfA % Positive**	**InfB % Positive**	**SC2 % Positive**
1000 copies/ µL	03	100%	100%	100%
100 copies/ µL	03	100%	100%	100%
50 copies/ µL	03	100%	100%	100%
25 copies/ µL	03	100%	100%	100%
12.5 copies/ µL	20	95%	100%	100%

References

1. Center for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories, 5th ed. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health HHS Publication No. (CDC) 21-1112, revised December 2009.
2. Clinical and Laboratory Standards Institute (CLSI). Protection of laboratory workers from occupationally acquired infections.
3. https://www.fda.gov/media/135659/download

Index of Symbols

IVD In Vitro Diagnostics only
Don't use if package is damaged
REF Catalogue Number
Expiry Date
Consult Instructions for use
Store at -20°C
LOT Batch Number
Manufacturer

CE-IVD

Manufactured by:
BioGenex Lifesciences Pvt. Ltd.
Plot No.4, survey #656/A,
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