Bio-Rad Reliance SARS-CoV-2/ FluA/FluB RT-PCR Assay Kit

Qualitative Assay for Use on Real-Time RT-PCR Instruments

Instructions for Use

For Rx Only

For Emergency Use Authorization Only

REF 12015361

SARS-CoV-2/FluA/FluB Oligos

Reliance One-Step Multiplex RT-qPCR Supermix

Exact Diagnostics SARS-CoV-2, Flu, RSV Positive Run Control

Exact Diagnostics SARS-CoV-2, Flu, RSV Negative Run Control

IVD

Bio-Rad Laboratories, Inc.
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10000135012 Rev. A

Translations

Product documents may be provided in additional languages on electronic media.

Symbols Lexicon

CE European Conformity	 Manufacturer	EC REP Authorized Representative in the European Union
LOT Lot Number	 Use by	IVD For In Vitro Diagnostic Use
 Temperature Limit	REF Catalog Number	 Consult Instructions for Use
 Number of Tests	USE For use with	SN Serial Number
Rx Only Prescription Use Only	UDI-DI Unique Device Identification – Device Identifier	LATEX Contains Latex
RUO Research Use Only	 Single Use Only	 Biohazard

Bio-Rad Technical Support

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific Time.

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For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact Us link at bio-rad.com.

Legal Notices

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Warnings and Precautions

For in vitro diagnostic use under Emergency Use Authorization. For healthcare professional use.

This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Wear appropriate protective clothing, gloves, and eye/face protection and handle appropriately with the requisite Good Laboratory Practices.

Personal Protective Equipment (PPE)

Proper use of gloves is recommended with the use of components and sample plates. OSHA requirements for PPE are set forth in the Code of Federal Regulations (CFR) at 29 CFR 1910.132 (General requirements); 29 CFR 1910.138 (Hand protection); 29 CFR 1926.95 (Criteria for standard personal protective equipment). Any gloves with impaired protective ability should be discarded and replaced.

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Intended Use

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the simultaneous qualitative detection and differentiation of nucleic acid from SARS-CoV-2, influenza A, and/or influenza B in nasopharyngeal swabs and anterior nasal swabs, collected from individuals suspected of respiratory viral infection consistent with COVID-19 by their healthcare provider. Clinical signs and 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, that meet requirements to perform high complexity tests.

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is intended for use in the simultaneous detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA in clinical 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 upper respiratory specimens during the acute phase of infection. Positive results are indicative of active infection but do not rule out bacterial infection or coinfection with other pathogens not detected by the test; 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 results to the appropriate public health authorities.

Negative Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit 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.

Testing with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is intended for use by qualified laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is only for use under the Food and Drug Administration's Emergency Use Authorization.

Summary and Principle

An outbreak of pneumonia caused by a novel coronavirus (SARS-CoV-2) in Wuhan City, Hubei Province, China, was identified and reported to the World Health Organization (WHO) on December 31, 2019. The rapid spread of SARS-CoV-2 to numerous areas throughout the world necessitates preparedness and response in healthcare and laboratory facilities. The availability of specific and sensitive assays for detecting the virus is essential for accurate diagnosis of cases, assessing the extent of the outbreak, monitoring of intervention strategies, and surveillance studies.

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is a molecular *in vitro* diagnostic test containing the reagents required to perform an RT-PCR test. The primer and probe sets are designed to detect and differentiate SARS-CoV-2, influenza A, and/or influenza B viral RNA in nasopharyngeal swabs and anterior nasal swabs, collected from individuals suspected of having respiratory viral infection

consistent with COVID-19 by their healthcare provider. Additional testing and confirmation procedures should be performed in consultation with public health and/or other authorities to whom reporting is required. Test results should be reported in accordance with local, state, and federal regulations. Performance is unknown in asymptomatic patients.

The oligonucleotide primers and probes for detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA are the same as those reported by the Center for Disease Control and Prevention (CDC) and contain three primer/probe sets (FluA, FluB, and SC2) that target the RNA of influenza A virus, influenza B virus, and SARS-CoV-2 virus, respectively. The assay also contains primers and a probe to detect the human RNase P gene (RP) in clinical specimens or control samples. The oligonucleotide primers and probe for detection of SARS-CoV-2 were selected from an evolutionarily conserved region of the 3' terminus of the SARS-CoV-2 genome and include part of the carboxy-terminal portion of the nucleocapsid (N) gene. Primers and probes for the detection of influenza A viruses were selected from an evolutionarily well-conserved region of the matrix (M1) gene. The primers and probe selected for the detection of influenza B viruses were selected from a conserved region of the nonstructural 2 (NS2) gene. The assay is a multiplex assay, run in a single well/vessel, designed to detect and differentiate RNA from SARS-CoV-2 virus, influenza A viruses, and influenza B viruses. An additional primer/probe set to detect the human RNase P (RP) gene in control samples and clinical specimens is also included in the panel. To perform a test, RNA is isolated and purified from control samples and clinical specimens then added to a master mix made using Bio-Rad Reliance One-Step Multiplex RT-qPCR Supermix. The master mix includes a reverse transcriptase that transcribes RNA into cDNA and a DNA polymerase that amplifies the cDNA fragments that share homology with the primer/probe sets. Amplification of specific targets is monitored by the change in fluorescence intensity within specific excitation/emission wavelengths using a real-time PCR instrument.

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit can be used with the Bio-Rad CFX Opus 96, CFX96 Touch, CFX96 Dx, and the Thermo Fisher Scientific, Inc. Applied Biosystems (AB) 7500 Fast Dx Real-Time PCR Systems (Table 1).

Table 1. Instruments Required

Catalog Number	Product Name
12011319	CFX Opus 96 Real-Time PCR System*
1855195	CFX96 Touch Real-Time PCR Detection System*
1845097-IVD 1841000-IVD	CFX96 Dx ORM C1000 Dx Thermal Cycler
4406985 or 4406984	Applied Biosystems 7500 Fast Dx Real-Time PCR System

**Starter packages available*

The CFX96 Touch and CFX Opus 96 Real-Time PCR Detection Systems utilize the CFX Maestro Software version 2.0. The CFX96 Dx utilizes the CFX Manager Dx Software version 3.1. The Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument utilizes software SDS version 1.4.

To mitigate cybersecurity risks, an environment in which access to the real-time instrument and computer are controlled by the persons who are responsible for the content of electronic records is essential. Bio-Rad requires the following security control to be applied to mitigate risk:

- Store the real-time instrument and computer in a secure location
- Enable Window's Firewall

- Enable AppLocker to prevent non-administrator users from running foreign software applications or scripts
- Enable Windows Virus & Threat Protection
- Enable user passwords to access the CFX Maestro and CFX Manager Dx
- Enable password-protected screensaver
- Disable unused ports, both inbound and outbound
- Disable Remote Services
- Disable Auto-run for the application
- Do not set Windows to log in to an Administrator account by default
- Install Windows Security Updates promptly
- Do not connect the computer to a network through either ethernet or WiFi (recommended)

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Workflow

The workflow consists of four steps (Table 2).

Table 2. Bio-Rad SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Workflow

Workflow	
Step 1	Isolation of viral RNA from nasal and mid-turbinate swab specimens as well as nasopharyngeal wash/aspirate and nasal aspirate specimen swabs
Step 2	RT-PCR Plate Setup
Step 3	One-step reverse transcription and qPCR
Step 4	Analysis

Reagents and Instruments

Materials Provided

The Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit contains sufficient reagents to process a total of 200 reactions (Table 3).

Table 3. Materials Included in the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit

Product Name	QTY (Tubes)	Volume (µL)	Storage Conditions, °C
Reliance One-Step Multiplex Supermix	1	1000	-20°C
Exact Diagnostics SARS-CoV-2, Flu, RSV Positive Run Control*	1	1000	-20°C
Exact Diagnostics SARS-CoV-2, Flu, RSV Negative Run Control*	1	1000	-20°C
SARS-CoV-2/FluA/FluB Oligos	1	300	-20°C

*The Reliance SARS-CoV-2/FluA/FluB RT-PCR Kit does not detect RSV and no results for RSV will be displayed when using the Positive and Negative Controls

Note: *Safety Data Sheets (SDS) are available at bio-rad.com*

Materials Required but Not Provided

Reagents and Consumables:

Reagents for RNA Purification

The QIAGEN QIAamp Viral Mini Kit (Catalog #52906, #52904) is validated for use with the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit per the manufacturer's instructions.

Generic Reagents and Consumables for Real-Time PCR

Materials required but not provided for running the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on the Bio-Rad and Thermo Fisher Scientific Real-Time PCR Systems are listed in Table 4 and Table 5.

Table 4. Materials Required but Not Provided for Running on the CFX Opus 96, CFX96 Touch, and CFX96 Dx Real-Time Systems

Bio-Rad Catalog #	Name	QTY (each)	Storage Conditions
MSB1001	Microseal 'B' PCR Plate Sealing Film, adhesive, optical	100	15°C to 30°C
HSP9955 or equivalent*	HSP9955, Hard-Shell 96-Well PCR Plates, low profile, thin wall, skirted, white/white	50	15°C to 30°C

***** Refer to Bio-Rad's Hard-Shell PCR Plate Brochure 5496 for other 96-well colored shell/white well PCR plates

Table 5. Materials Required but Not Provided for Running on the Applied Biosystems 7500 Fast Dx Real-Time PCR System

Thermo Fisher Scientific Catalog #	Product Name	QTY (each)	Storage Conditions
4311971	MicroAmp Optical Adhesive Film	100	15°C to 30°C
4346906	MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	20	15°C to 30°C

Instrumentation, Software, and General Laboratory Equipment:

General laboratory equipment required but not provided for running the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is listed in Table 6.

Table 6. General Laboratory Equipment Required but Not Provided

Description	Source
Single and multichannel adjustable pipettors (1.00 µL to 1,000.0 µL)	Rainin or Eppendorf
Microcentrifuge	Multiple suppliers
Microwell plate centrifuge, with a rotor that accommodates standard microplates	Multiple suppliers
Laboratory mixer, vortex, or equivalent	Multiple suppliers
Laboratory freezers • –30°C to –10°C • ≤ –70°C	Multiple suppliers
96-well cold block or ice	Multiple suppliers
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	Multiple suppliers
Sterile aerosol barrier (filtered) pipette tips	Multiple suppliers

General Precautions and Warnings

1. For In vitro Diagnostic (IVD) Use under Emergency Use Authorization only.
2. For Prescription (Rx) Use only
3. For professional use only.
4. This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use in laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C.§263a, that meet requirements to perform high complexity tests;
5. This product has been authorized only for the simultaneous qualitative detection and differentiation of nucleic acids from SARS-CoV-2, influenza A virus, and influenza B virus, and not for any other viruses or pathogens; and,
6. The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the declaration is terminated or authorization is revoked sooner.
7. All biological specimens should be treated as if they are capable of transmitting infectious agents. Use safe laboratory procedures, such as those outlined in HHS Publication (CDC) 21-1112, Biosafety in Microbiological and Biomedical Laboratories, and in CLSI Document M29-A4, Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue. [1, 2]
8. Specimens should be collected using appropriate infection control precautions if infection with SARS-CoV-2 is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities. If infection with a novel influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, for novel virulent influenza viruses and sent to state health departments for testing.
9. Positive SC2 results are indicative of the presence of SARS-CoV-2 RNA. Laboratories within the United States and its territories should report all SARS-CoV-2 test results to the appropriate public health authorities, as required.
10. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite (10% bleach) in deionized or distilled water, followed by 70% alcohol. CAUTION: Lysis buffers used with the QIAamp extraction methods contain guanidinium thiocyanate or guanidine containing materials. Highly reactive and/or toxic compounds may form if combined with sodium hypochlorite (bleach).
11. To minimize nucleic acid contamination, routinely decontaminate bench space, pipettors, and equipment, and separate the specimen and RNA/DNA handling area from the assay preparation area.
12. Optimize workflow and space to minimize the risk of carryover contamination from completed PCR reactions.
13. Ensure that the real-time PCR system and automation system have a dedicated space in separate areas to avoid amplicon contamination.
14. Perform assay setup and template addition in different locations with dedicated pipettors.
15. Use proper laboratory safety procedures for working with chemicals and handling specimens.
16. Change gloves frequently when transporting and working with different reagents.
17. Failure to follow the procedures and conditions described in this document can cause incorrect results and adverse effects.

18. Do not substitute Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit reagents with other reagents.
19. Setup and template addition must be performed under RNase/DNase-free conditions.
20. Ensure that regular maintenance and calibration are performed on all equipment according to the manufacturer's recommendations.
21. Use nuclease-free tips and reagents, and routinely clean pipettors.
22. Ensure that only the recommended thermal cycling protocol is used.
23. Do not use diethylpyrocarbonate (DEPC) treated water for PCR amplification.
24. Closely follow the procedures and guidelines provided to ensure that the test is performed correctly. Any deviation from the procedures and guidelines may affect optimal test performance.
25. False-positive results may occur if the carryover of samples is not adequately controlled during sample handling and processing.
26. Individuals who received nasally administered influenza A/B vaccine may have positive influenza test results for up to three days after vaccination. http://www.cdc.gov/mmwr/preview/mmwrhtml/rr57e717a1.htm

Specimen Collection, Handling, and Storage

Adequate, appropriate specimen collection, storage, and transport are important to obtain sensitive and accurate test results. Training in correct specimen collection procedures is highly recommended to ensure good quality specimens and results. CLSI MM13-Ed2 may be referenced as an appropriate resource.

1. Sample acceptance criteria
   - Samples should be collected into sterile, labeled tubes and shipped per testing laboratory requirements.
2. Specimen rejection criteria
   - Samples that have not been pre-approved for testing and those that are labeled improperly will not be tested until the required information is obtained.
3. Collecting the specimen
   - Refer Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19). https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
   - Follow manufacturer instructions for proper use of specimen collection devices.
   - Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable, and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 2-3 mL of viral transport media or universal transport media.
4. Transporting specimens
   - Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens to the testing laboratory.
   - Store specimens at 2-8°C and ship overnight to the testing laboratory on an ice pack. If a specimen is frozen at -70°C or lower, ship overnight to the testing laboratory on dry ice.
5. Storing specimens
   - Specimens can be stored at 2-8°C for up to 72 hours after collection if necessary.

- If a delay in extraction is expected, store specimens at -70°C or lower until processing can commence.
- Extracted nucleic acid should be stored at 4°C if it is to be used within 4 hours, or -70°C or lower if material must be stored longer than 4 hours.

Use of Control Materials

Controls to be used with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit:

- A no-template control (NTC) is needed to detect reagent and/or environmental contamination. This control uses RNase/DNase-free water in place of a clinical specimen sample and should be present in a minimum of one well per reaction plate.
- A positive control is needed to detect substantial reverse transcription and/or reagent failure, including primer and probe integrity. The test utilizes Exact Diagnostics SARS-CoV-2, Flu, RSV Positive Run Control, which is manufactured with whole inactivated influenza A, influenza B, RSV, and SARS-CoV-2 viruses. One positive control must be included per batch of samples extracted, with a minimum of one positive control well per reaction plate. The Reliance SARS-CoV-2/FluA/FluB assay does not detect RSV and no result is obtained for this analyte with the Positive Control.
- A negative control is needed to detect extraction step failure or reagent/environmental contamination. The test utilizes Exact Diagnostics SARS-CoV-2, Flu, RSV Negative Run Control, which is manufactured with human genomic DNA and RNA. One negative control must be included per batch of samples extracted, with a minimum of one negative control well per reaction plate. The Reliance SARS-CoV-2/FluA/FluB assay does not detect RSV and no result is obtained for this analyte with the Negative Control.

Reagent Handling and Storage

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit

- The kit contains RT-PCR supermix, assay oligos, positive and negative controls
- Storage at -20°C is recommended, with minimum freeze-thaw cycles

Work Areas

All necessary safety precautions should be taken according to the laboratory guidelines. Precautions must also be taken to prevent cross-contamination of samples.

Separate work areas should be used for:

- Nucleic acid extraction
- Reagent preparation (for example, preparation of master mix)
    - No amplified reactions, target solutions, or clinical specimens should be brought into the reagent preparation area. After working in this area, laboratory coat and gloves should be changed before moving into the nucleic acid addition area
- Nucleic acid addition
- Instrumentation (for example, thermocyclers)

General Handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from the surface of the skin or laboratory equipment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything on cold blocks to avoid degradation of RNA by endogenous or residual RNases. Clean working surfaces, pipettes, etc., with 10% bleach or other solutions that can destroy nucleic acids and RNases. To eliminate accelerated deterioration of any plastics and metals, wipe down with 70% ethanol after using 10% bleach. Ensure all bleach is removed to eliminate possible chemical reactions between bleach and guanidine thiocyanate, which is present in the extraction reagents.

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Protocol

Overview

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A, and/or influenza B viral RNA in nasopharyngeal swabs and anterior nasal swabs, collected from individuals suspected of having respiratory viral infection consistent with COVID-19 by a healthcare provider. The assay contains primer/probe sets (FluA, FluB, and SC2) targeting viral RNA of influenza A, influenza B, and SARS-CoV-2. An additional human gene, Human RNase P (RP), is included in the assay as an internal control. Detection of viral RNA aids in the diagnosis of illness and provides epidemiological and surveillance information.

The test comprises two main steps: (1) extraction of RNA from patient specimens and (2) one-step reverse transcription and polymerase chain reaction amplification and detection of the targets.

Description of Test Steps

Nucleic acids are isolated and purified from nasopharyngeal swabs and anterior nasal swabs using the QIAGEN QIAamp Viral RNA Mini Kit, following the manufacturer's instructions. The purified nucleic acids are reverse transcribed then amplified using Reliance One-Step Multiplex RT-qPCR Supermix into cDNA in a real-time PCR instrument (Table 1). The thermocycling protocol used on all instruments is 50.0°C for 10 min, 95.0°C for 10 min, and 45 cycles of (95.0°C for 10 sec, 60.0°C for 30 sec, plate read). The Reliance One-Step Multiplex RT-qPCR Supermix contains the reverse transcriptase enzyme and thermostable DNA polymerase that supports cDNA synthesis and probe-based qPCR in one tube. The SARS-CoV-2/FluA/Flu/B Oligos contain the primers and probes for SARS-CoV-2, influenza A, and influenza B targets, as well as the human RP gene for multiplex qPCR. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the PCR cycle extension phase, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. Additional reporter dye molecules are cleaved from their respective probes with each cycle, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by real-time PCR instruments.

Nucleic Acid Extraction

Performance of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit depends on the amount and quality of template RNA purified from human specimens. The QIAGEN QIAamp Viral RNA Mini Kit (Catalog #52906, #52904) extraction kit has been qualified and validated for recovery and purity of RNA for use with the test.

Follow the manufacturer's recommended procedures for sample extraction. Positive and negative controls must be included in each extraction batch.

Preparation of controls

Positive control: Extract 140 µL of Exact Diagnostics SARS-CoV-2, Flu, RSV Positive Run Control using QIAGEN QIAamp Viral RNA Mini Kit together with patient samples according to the manufacturer's instructions.

Negative control: Extract 140 µL of Exact Diagnostics SARS-CoV-2, Flu, RSV Negative Run Control using QIAGEN QIAamp Viral RNA Mini Kit together with patient samples according to the manufacturer's instructions.

Preparation of One-Step RT-PCR Reaction

1. Ensure extracted RNA sample(s) are thawed on ice.

   Note: *Do not vortex RNA samples. RNA samples may be mixed by flicking the tubes, followed by brief centrifugation to collect the contents to the bottom of the tubes.*

2. Thaw all kit components on ice.
3. Mix thoroughly by vortexing each tube briefly to ensure homogeneity, then pulse centrifuge to collect contents at the bottom of each tube.

   Note: The Reliance One-Step Multiplex RT-qPCR Supermix is viscous and will not freeze at -20°C. Some gelling and precipitation may occur; this is expected and does not impact the reagent's stability or performance. It is important to thoroughly vortex the reagent before beginning the master mix preparation.
4. RT-PCR master mix preparation:
   a. Prepare a master mix volume sufficient for the number of patient samples and controls to be tested plus 10% more volume (Table 7) when more than 1 sample is being tested.
   b. Vortex the master mix briefly and pulse centrifuge to collect the contents to the bottom of the tube.

Table 7. RT-PCR Master Mix Component Volumes

Component	Volume for 1 Sample (µL)	Volume for 96 Samples (µL)	Volume for N Samples (µL)
Reliance One-Step Multiplex RT-qPCR Supermix	5.0	528	(5.0 x N) x 1.1
SARS-CoV-2/FluA/FluB Oligos	1.5	158	(1.5 x N) x 1.1
RNase/DNase free water	3.5	370	(3.5 x N) x 1.1
Volume per reaction	**10.0**	**1056**	**(10.0 x N) x 1.1**

5. Dispense 10 µL of the master mix into the appropriate wells of the RT-PCR plate.
6. Add 10 µL of RNase/DNase free water to one well for an NTC.
7. Add 10 µL of negative control material to one well for a Negative Control.

8. Add 10 µL of positive control material to one well for a Positive Control.
9. For the remaining wells, add 10 µL of extracted RNA sample per well.
10. Seal the plate with an optical sealing film.
11. Vortex the plate for 30 seconds at high speed.
12. Centrifuge the RT-PCR reaction plate for 30 seconds at 1000 RCF to remove any air bubbles and allow the RT-PCR reaction to settle to the bottom of the wells. If bubbles remain, spin the plate again.
13. Proceed with loading the RT-PCR reaction plate onto a Bio-Rad CFX Opus 96, CFX96 Touch, CFX96 Dx Real-Time PCR Detection System, or an Applied Biosystems 7500 Fast Dx Real-Time PCR instrument.

Bio-Rad CFX Instrument Setup

The following instructions are for running SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on a Bio-Rad CFX Opus 96, CFX96 Touch, or CFX96 Dx Real-Time PCR Detection System. For more detailed information, refer to the instrument manual.

Using Bio-Rad CFX instrument software, there are three stages for an RT-PCR run:

1. Protocol Setup
2. Plate Setup
3. Running the RT-PCR reaction

Cycling Protocol Setup

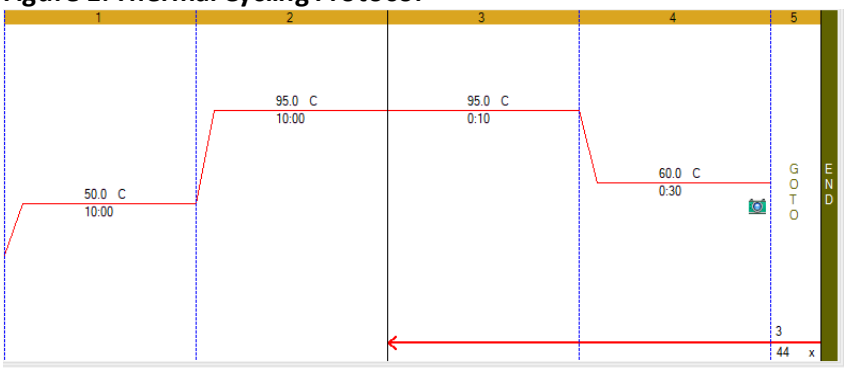
1. Click **File** -> **New** -> **Protocol** in the menu bar to open the Protocol Editor
2. Change Sample Volume to 20 µl
3. Modify the Cycling Protocol to the guidelines in Table 8

Table 8. Thermal Cycling Protocol

Step Number	Cycling Step	Temperature (°C)	Time	Cycles
1	Reverse transcription	50	10 minutes	1
2	Enzyme activation	95	10 minutes	1
3	Denaturation	95	10 seconds	45
4	Annealing/extension/ plate read	60	30 seconds	
5	Go to step 3 and repeat 44 times	--	--	

4. Confirm step 4 includes a plate read, as is indicated by a camera symbol in the step
5. To add a plate read to step 4, click on the step to highlight, then click **Add Plate Read to Step**

Figure 1: Thermal Cycling Protocol



6. Save the protocol by clicking **File -> Save As**
7. Name the protocol file "**Bio-Rad SARS-CoV-2_FluA_FluB RT-PCR Protocol**"

Plate Setup

1. Click **File** -> **New** -> **New Plate** in the menu bar to open the Plate Editor
2. Select **Settings** -> **Plate Size** -> select 96 wells
3. Select **Settings** -> **Plate Type** -> select BR White
4. Expand the pull-down menu to the right of **Scan Mode** and select All Channels
5. Highlight the wells where samples and controls will be on the plate. To highlight all wells, click the upper left corner of the plate graphic.
6. Click **Select Fluorophores** and select FAM, HEX, Texas Red, and Cy5 by checking the Selected box to the right of the fluorophore (uncheck SYBR). Click OK to apply changes.
7. Define sample type for each well by highlighting the wells then choose the appropriate identifier from the Sample Type pull-down menu **Wells** -> **assign Sample Type**
8. Apply **target names and fluorophores** to all wells by highlighting the wells then checking the Load box to the left of each of the fluorophores listed in the Target Name section. To include the target name, replace <none> in the open text box to the right of the fluorophore with the following:
   FAM – SARS-CoV-2
   HEX – Influenza A
   Texas Red – RNase P
   Cy5 – Influenza B
9. Save the file by clicking **File -> Save As**
10. Name the plate file "**Bio-Rad SARS-CoV-2_FluA_FluB RT-PCR Plate Setup**"
11. Close the file by clicking **File -> Close**

Running the RT-PCR Plate on CFX Real-Time PCR Systems

1. Select the instrument from the Select Instrument drop-down menu in the Startup Wizard
2. Click User-defined in the Select run type section of the Startup Wizard. This will open the Run Setup panel.
3. Click **Select Existing** in the protocol tab
4. Select the cycling protocol file "**Bio-Rad SARS-CoV-2_FluA_FluB RT-PCR Protocol.prcl"**
5. Click Open to apply
6. Confirm the cycling protocol is as shown in Table 8
7. Click the Plate tab in the Run Setup panel

8. Click **Select Existing**
9. Select the plate setup file "**Bio-Rad SARS-CoV-2_FluA_FluB RT-PCR Plate Setup.pltd"**
10. Click Open to apply
11. Click the Start Run tab in the Run Setup panel
12. Select the instrument in the Start Run on Selected Blocks section by checking the box to the left of the instrument name
13. Load the plate into the instrument
14. Click **Start Run**
15. Define a file name for the run file and click Save to begin the run

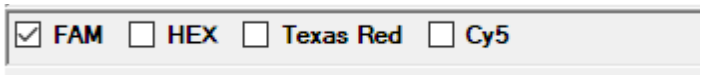
Data Analysis on CFX Real-Time PCR Systems

The run data file will open automatically after the run. To open a file that has been closed, click **File** -> **Open** -> **Data File** -> Select the data file from the menu.

To analyze the data, adjust the baseline and threshold values for each fluorophore in the Quantification Tab.

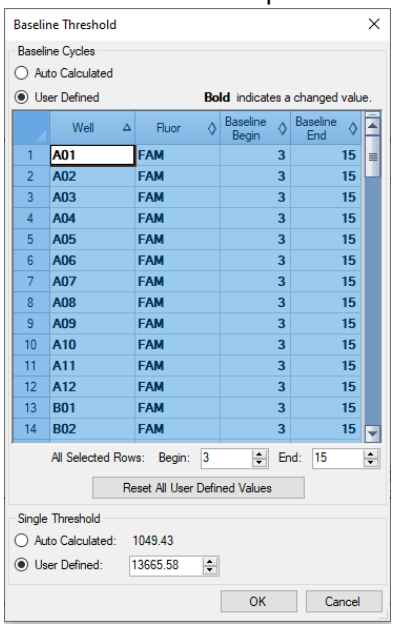
1. Select **Settings > Cycles to Analyze** and enter "5" in the first cell
2. Select all wells by clicking the top-left corner of the plate map below the amplification plot
3. Right-click anywhere in the amplification plot and select **Chart Settings.** Click **Axis Scale** tab and uncheck the **Auto Scale** button. Click **OK** to apply.
4. Select Log Scale by checking the box in the lower right of the amplification plot

5. Deselect **HEX**, **Texas Red**, and Cy5 fluorophores by unchecking the corresponding boxes under the amplification plot. Only the FAM box should be selected

6. Drag the bold, horizontal threshold line so that it intersects with the highest-amplitude amplification trace at cycle 45
7. Manually define the baseline for all traces. Select **Settings > Baseline Threshold.** Highlight all wells by clicking on the top-left corner of the table. Below the table, apply the following for All Selected Rows: Begin: 3 and End: 15

8. Set the FAM threshold to 10% of the maximum amplitude trace signal. Confirm that the 'User Defined' radio button is selected. Set the value to 10% of that shown (e.g., the initial value of 13665.58 in the example below would become 1366.558). Click **OK** to apply.



9. Adjust baseline and define the threshold for each of the HEX, Texas Red, and Cy5 channels by selecting the appropriate fluorophore in Step 1 and repeating Step 5 through Step 8.

Applied Biosystems 7500 Fast Dx Real-Time Instrument Setup

The following instructions are essential for testing SARS-CoV-2/FluA/FluB RT-PCR Assay with the 7500 Fast Real-Time PCR system. For more detailed information about how to set up plates and cycling protocols, refer to the Applied Biosystems 7500 Fast Real-Time PCR instrument manual.

1. Launch the 7500 software
2. Select **File** -> **New** from the menu bar
3. Define the following
   a. **Assay – Standard Curve (Absolute Quantitation)**
   b. **Container – 96-Well Clear**
   c. **Template – Blank Document**
   d. **Run Mode – Standard 7500**
4. Assign the report dye as defined in Table 9.

Table 9: Required Reporter Dyes

Reporter Dye	Detector
FAM	SARS-CoV-2
HEX	Influenza A
TEXAS RED (TRed)	RNase P
Cy 5	Influenza B

Note: For the Inf A assay, the VIC channel is applicable for detecting this target.

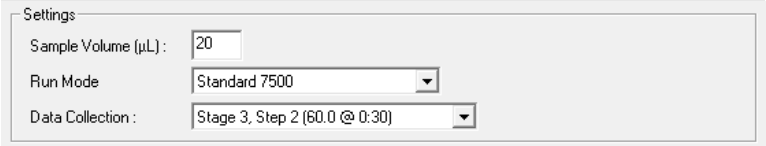
5. Select **Passive Reference** -> **None**
6. Define the cycling protocol using the values listed in Table 10.

Table 10. Thermal Cycling Protocol for AB7500

Cycling Step	Temperature (°C)	Time	Number of Cycles
Reverse transcription	50	10 minutes	1
Enzyme activation	95	10 minutes	1
Denaturation	95	10 seconds	45
Annealing/extension	60	30 seconds	

7. Define the data collection step by selecting Stage 3, step 2 (60.0 @ 0:30) from the Data Collection dropdown menu, select Stage 3, Step 2 (60.0 @ 0:30), see Figure 2

Figure 2: Data Collection Dropdown Menu for AB7500 Dx



Data Analysis on Applied Biosystems 7500 Dx Fast Real-Time

The following instructions are essential for analyzing the SARS-CoV-2 RT-PCR with the 7500 Fast Dx Real-Time PCR system. For more detailed information about data analysis, refer to the Applied Biosystems 7500 Fast Real-Time PCR instrument manual.

Setting Baseline and Threshold Values

1. Select **File** -> **Open** -> Select the data file to be analyzed
2. Select **Analysis Settings** to open the "Analysis Setting-Standard curve" dialog box.
    a. Select **All** under **Detector**. Select **Manual Baseline**. Make sure the **Start (cycle)** is **3** and the **End (cycle)** is **15**.
    b. Click **OK and Reanalyze** to close the dialog box
3. Find the maximum RFU value in each channel for the entire plate by following the steps below:
    a. Select all wells
    b. Select **File** -> **Export** -> **Delta Rn** and save the csv file.
    c. Open the csv file in step b using Excel and select all cells by clicking on the upper left corner of the spreadsheet.
    d. Select **Insert -> PivotTable**. Click OK of the dialog box without changing any of the default setting
    e. In the PivotTable Fields, drag Detector to the Rows area and Cycle 45 to the Values area.
    f. Click "Sum of Cycle 45" in the Values box (lower right).
    g. Select "Value field settings" and change to "Max"
    h. Make note of the maximum RFU value for each channel (data presented in pivot table)
4. Return to the RT-PCR run file. Select **Analysis Settings** to open the "Analysis Setting-Standard curve" dialog box.
    a. Select **FAM** under **Detector**.
    b. Select **Manual Ct**
    c. Enter **10%** of the value of the maximum RFU noted for the FAM channel in step 3-h.

d. Repeat a-c for the HEX, TRed, and Cy5 channels using the % Maximum RFU shown in Table 11.

Table 11. Percent Maximum RFU to Define Threshold Value

Channel	% Maximum RFU
FAM	10%
HEX	5%
TRed	10%
Cy5	10%

e. Click **OK and Reanalyze** to close the dialog box.

Interpretation of Results

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

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Controls – NTC, Positive and Negative

No Template Control (NTC)
The amplification curves for the NTC reactions should not cross the threshold before cycle 40 in any channel (FAM, HEX, Cy5, or Texas Red). If any of the NTC reactions cross the threshold before cycle 40, sample contamination may have occurred. Invalidate the run and repeat the assay with the residual extracted nucleic acid with strict adherence to the guidelines. If the repeat test result is positive, re-extract, and retest all samples included in that batch.

Negative Control (NC)
The amplification curves for the SARS-CoV-2, Flu, RSV Negative Run Control must cross the threshold before cycle 40 (Cq < 40) in the Texas Red channel (RP) and fail to cross or cross the threshold after cycle 40 in the FAM, HEX, and Cy5 channels. If the NC fails, the run is repeated with residual extracted nucleic acid (all samples, NTC, PC and, NC). If the NC fails again, the extraction must be repeated for all samples included in that batch.

Positive Control (PC)
The amplification curves for the SARS-CoV-2, Flu, RSV Positive Run Control must cross the threshold before cycle 40 (Cq < 40) in all four channels (FAM, HEX, Cy5, and Texas Red). If the PC fails, the run is repeated with residual extracted nucleic acid (all samples, NTC, PC and, NC). If the PC fails again, the extraction must be repeated for all samples included in that batch.

Examination and Interpretation of Patient Specimen Results

Assessment of clinical specimen test results must be performed after the positive and negative controls have been confirmed. The expected performance of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit controls is shown in Table 12.

Table 12. Expected Performance of Controls in the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit

Control Type	External Control Name	Used to Monitor	FluA	FluB	SC2	RP	Expected Cq
NTC	RNase/DNase free water	Reagent and/or environmental contamination	Negative	Negative	Negative	Negative	Cq ≥40.00 for FluA, FluB, SC2, and RP
Negative	SARS-CoV-2, Flu, RSV Negative Run Control	Reagent and/or environmental contamination; extraction failure	Negative	Negative	Negative	Positive	Cq < 40.00 for RP Cq ≥40.00 for FluA, FluB, and SC2
Positive	SARS-CoV-2, Flu, RSV Positive Run Control	Substantial reagent failure including primer and probe integrity	Positive	Positive	Positive	Positive	Cq < 40.00 for FluA, FluB, SC2, and RP

If any control does not meet these criteria, the test may have been improperly set up or executed, or reagent or equipment may have experienced malfunction or failure. Invalidate the run and retest according to the above procedures.

RP (Internal Control)

All clinical samples must exhibit positive signals with the RP primers and probe (Cq < 40) in the Texas Red channel, indicating the presence of the human RP gene. Failure to detect RP in any clinical specimens may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity
- Improper assay set up and execution
- Reagent or equipment malfunction

If the RP assay does not produce a positive result for a human clinical specimen, interpret as follows:

- If any of the FluA, FluB, and SC2 targets is/are positive, even in the absence of a positive RP, the result should be considered valid. Some samples may fail to exhibit RP as positive (Cq < 40) due to low cell numbers in the original clinical sample. A negative RP signal does not preclude the presence of FluA, FluB, and SC2 viral RNA in a clinical specimen.
- If all FluA, FluB, and SC2 markers and RP are negative for the specimen, the result should be considered invalid. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after retesting, report the results as invalid and a new specimen should be collected.

FluA, FluB, and SC2 Markers

When positive, negative, and NTC controls exhibit the expected results:

- Positive result: One or more of the viral amplification curves (FluA, FluB, and/or SC2) crosses the threshold BEFORE 40 cycles (Cq < 40.00). Multiple viruses may be detected in a single specimen.
- Negative result: All viral amplification curves (FluA, FluB, and SC2) fail to cross the threshold or cross the threshold AFTER 40 cycles (Cq ≥ 40.00) AND the RP amplification curve crosses the threshold BEFORE 40 cycles (Cq ≤ 40.00).

- Invalid result: Amplification curves for FluA, FluB, and SC2 fail to cross the threshold or cross the threshold AFTER 40 cycles (Cq ≥ 40.00) but the RP also fails to cross the threshold BEFORE 40 cycles (Cq < 40.00). Repeat testing of the specimen nucleic acid and/or re-extract and repeat the assay. If the specimen remains invalid upon retest then the collection of a new specimen and subsequent testing should be considered.

For ease of interpretation, refer to the guide in Table 13.

Table 13. Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Results Interpretation Guide

FluA Result	FluB Result	SC2 Result	RP Result	Interpretation	Actions
Positive (Cq < 40)	**Negative** (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Positive or Negative**	Influenza A detected	Report results to sender and appropriate public health authorities.
Negative (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	**Negative** (Cq ≥ 40 or N/A)	**Positive or Negative**	Influenza B detected	Report results to sender and appropriate public health authorities.
Negative (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	**Positive or Negative**	SARS-CoV-2 detected	Report results to sender and appropriate public health authorities.
Positive (Cq < 40)	**Positive** (Cq < 40)	**Negative** (Cq ≥ 40 or N/A)	**Positive or Negative**	Influenza A and Influenza B detected	Report results to sender and appropriate public health authorities.
Positive (Cq < 40)	**Negative** (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	**Positive or Negative**	Influenza A and SARS-CoV-2 detected	Report results to sender and appropriate public health authorities.
Positive (Cq < 40)	**Positive** (Cq < 40)	**Positive** (Cq < 40)	**Positive or Negative**	Influenza A, Influenza B, and SARS-CoV-2 detected	Report results to sender and appropriate public health authorities.
Negative (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	**Positive** (Cq < 40)	**Positive or Negative**	Influenza B and SARS-CoV-2 detected	Report results to sender and appropriate public health authorities.
Negative (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	Virus not detected	Report results to sender and appropriate public health authorities. Consider testing for other respiratory viruses.
Negative (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	Invalid	Repeat extraction and RT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

N/A; Negative, no detectable Cq value

Limitations

1. Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit has only been evaluated for use in combination with the SARS-CoV-2 RT-PCR Oligos, Reliance One-Step Multiplex RT-qPCR Supermix, and the Exact Diagnostics SARS-CoV-2, Flu, RSV Positive and Negative Run Controls for use on the CFX Opus 96, CFX96 Touch, CFX96 Dx, and Applied Biosystems 7500 Fast DX Real-Time PCR Systems.
2. The clinical performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation.

Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.

3. Reliable results depend on proper sample collection, storage, and handling procedures.
4. This test is used for the detection of viral RNA in nasopharyngeal swabs and anterior nasal swabs collected in a Universal Transport Medium (UTM) or Universal Viral Transport System (UVT). Testing of other sample types with Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit may result in inaccurate results.
5. Detection of viral RNA may be affected by sample collection methods, patient factors (for example, presence of symptoms), and/or stage of infection.
6. The output of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit is a qualitative assessment of positive patient samples. The user assesses RT-PCR results for controls and patient samples to make a qualitative call of viral RNA detected or not detected. The values reported should not be used or interpreted as quantitative.
7. As with any molecular test, mutations within the target regions of Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit could affect primer and/or probe binding resulting in a failure to detect the presence of a virus.
8. Due to inherent differences between technologies, it is recommended that users perform method correlation studies in their laboratory to qualify technology differences before switching from one technology to the next. One hundred percent agreement between the results should not be expected due to the aforementioned differences between technologies. Users should follow their own specific policies/procedures.
9. Individuals who received nasally administered influenza A/B vaccine may have positive influenza test results for up to three days after vaccination.
   http://www.cdc.gov/mmwr/preview/mmwrhtml/rr57e717a1.htm

Conditions of Authorization for the Laboratory

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website: https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas.

However, to assist clinical laboratories using the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit, ("your product" in the conditions below), the relevant Conditions of Authorization are listed below:

A. Authorized laboratories[1] using your product must include with test result reports all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.

B. Authorized laboratories using your product must use your product as outlined in the authorized labeling. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use your product are not permitted.

C. Authorized laboratories that receive your product must notify the relevant public health authorities of their intent to run your product prior to initiating testing.

D. Authorized laboratories using your product must have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

E. Authorized laboratories must collect information on the performance of your product and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and Bio-Rad Technical Support at 1-800-4BIORAD (1-800-424-6723) about any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of your product of which they become aware.

F. All laboratory personnel using your product must be appropriately trained in PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit and use your product in accordance with the authorized labeling.

G. You, authorized distributors, and authorized laboratories using your product must ensure that any records associated with this EUA are maintained until otherwise notified by the FDA. Such records will be made available to the FDA for inspection upon request.

[1] The letter of authorization refers to, "Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests" as "authorized laboratories."

Analytical Performance Characteristics

Analytical Sensitivity

LoD Using Live Influenza Viruses

To determine the sensitivity of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit to detect low viral loads, a limit of detection (LoD) study was conducted. An initial test was performed to identify the viral titer range to conduct LoD studies. The LoD of each primer/probe set was determined using contrived samples prepared by titrating each virus separately into a background of pooled SC2/FluA/FluB negative nasopharyngeal swab matrix before nucleic acid purification. The viral strains used and stock concentration are described in Table 14. For each dilution point, 5 replicates were extracted using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume), and the nucleic acid from these extractions was tested immediately with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on the CFX96 Touch Real-Time PCR Instrument. Results from the range-finding runs, shown in Table 15, indicate that the lowest level at which all replicates are positive for influenza A is 0.618 $TCID_{50}$/mL, for influenza B is 1.07 $TCID_{50}$/mL and for SARS-CoV-2 is 953 copies (cp)/mL.

Table 14. Stock Viral Strains used for LoD Studies

Virus	Strain	Source	Catalog No.	Stock Concentration
Influenza A*	H1N1 (A/PR/8/34)	ATCC	VR-1469	15.81E06 $TCID_{50}$/mL 6.26E09 cp/mL
Influenza B*	(Lee/40)	ATCC	VR-1535	2.81E06 $TCID_{50}$/mL 1.03E10 cp/mL
SARS-CoV-2	2019-nCoV/USA-WA1/2020	ATCC	VR-1986HK	2.44E08 cp/mL

**Live cultured virus*

Table 15. FluA, FluB and SC2 LoD Range Finding

Virus	$TCID_{50}$ / mL	#Positive	Rep 1 (Cq)	Rep 2 (Cq)	Rep 3 (Cq)	Rep 4 (Cq)	Rep 5 (Cq)
Influenza A	9.88	5/5	31.98	32.06	32.30	31.72	32.10
	2.47	5/5	34.42	34.05	33.55	34.02	35.32
	0.618	5/5	35.29	36.62	37.51	38.09	36.50
	0.154	2/5	37.65	37.71	N/A	N/A	N/A
	0.0386	1/5	37.51	N/A	N/A	N/A	N/A
Influenza B	68.6	5/5	26.68	26.64	26.92	26.87	27.19
	8.58	5/5	30.57	30.81	30.96	30.33	30.55
	1.07	5/5	33.30	33.25	33.15	33.74	32.81
	0.1341	3/5	34.61	37.01	35.27	N/A	N/A
	0.0168	0/5	N/A	N/A	N/A	N/A	N/A
Virus	**cp/mL**	**#Positive**	**Rep 1 (Cq)**	**Rep 2 (Cq)**	**Rep 3 (Cq)**	**Rep 4 (Cq)**	**Rep5 (Cq)**
SARS-CoV-2	488,000	5/5	28.00	28.15	28.21	28.07	28.04
	61,000	5/5	31.26	31.18	31.17	31.41	31.42
	7,625	5/5	34.91	34.90	34.41	34.35	34.36
	953	5/5	37.37	39.08	39.32	38.32	37.29
	119	1/5	N/A	N/A	N/A	39.85	N/A
	14.9	0/5	N/A	N/A	N/A	N/A	N/A

N/A; Negative, no detectable Cq value

Limit of Detection (LoD) - Confirmation

The FluA, FluB, and SC2 LoD were determined using the same contrived sample procedure described for the range-finding study. A 2-fold dilution series was performed based on the range-finding study. For each dilution point, 20 replicates were extracted using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume). The nucleic acid from these extractions was tested immediately with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on the CFX Opus 96, CFX96 Touch, CFX96 Dx, and AB 7500 Fast Dx. The LoD was determined as the lowest amount of virus detected, with at least 95% of the replicates testing positive.

Results are presented in Table 16, Table 17, and Table 18 and are summarized in Table 19. All controls performed as expected. The LoD for SARS-CoV-2 ranges from 125 to 250 cp/mL on the different instruments. The LoD for influenza A ranges from 1.26 to 2.52 $TCID_{50}$/mL on the different instruments. The LoD for influenza B is 0.23 $TCID_{50}$/mL on all instruments. Each virus has equivalent LoD performance (within 2-3 fold) across all validated instruments with the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit.

Table 16. Limit of Detection of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit in Detecting the SARS-CoV-2 Virus

SARS-CoV-2 copies/mL	CFX96 Touch	CFX96 Dx	CFX Opus 96	AB7500 Fast Dx
	SC2 Positive replicates/Total replicates	SC2 Positive replicates/Total replicates	SC2 Positive replicates/Total replicates	SC2 Positive replicates/Total replicates
250	19/20	20/20	20/20	19/20
125	19/20	14/20	15/20	13/20
62.5	12/20	11/20	14/20	15/20
31.25	5/20	7/20	13/20	6/20

Table 17. Limit of Detection of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit in Detecting the FluA Virus

FluA $TCID_{50}$/mL	CFX96 Touch	CFX96 Dx	CFX Opus 96	AB7500 Fast Dx
	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates
5.04	20/20	20/20	20/20	20/20
2.52	19/20	20/20	20/20	20/20
1.26	11/20	18/20	15/20	19/20
0.63	8/20	14/20	14/20	18/20

Table 18. Limit of Detection of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit in Detecting the FluB Virus

FluB $TCID_{50}$/mL	CFX96 Touch	CFX96 Dx	CFX Opus 96	AB7500 Fast Dx
	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates
0.92	20/20	20/20	20/20	20/20
0.46	20/20	19/20	19/20	19/20
0.23	20/20	20/20	20/20	20/20
0.12	17/20	18/20	16/20	15/20

Table 19. Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit LoD Summary

Instrument	SARS-CoV-2	FluA*		FluB*	
CFX96 Touch	125 copies/mL	2.52 $TCID_{50}$/mL	1002 copies/mL	0.23 $TCID_{50}$/mL	862 copies/mL
CFX96 Dx	250 copies/mL	2.52 $TCID_{50}$/mL	1002 copies/mL	0.23 $TCID_{50}$/mL	862 copies/mL
CFX Opus 96	250 copies/mL	2.52 $TCID_{50}$/mL	1002 copies/mL	0.23 $TCID_{50}$/mL	862 copies/mL
AB7500 Fast Dx	250 copies/mL	1.26 $TCID_{50}$/mL	501 copies/mL	0.23 $TCID_{50}$/mL	862 copies/mL

**Live cultured virus*

The interfering substances study used live contemporary influenza A (A/Kansas/14/2017) and influenza B (Colorado/06/2017 [Victoria]) strains. An additional confirmatory LoD study was performed on these strains to confirm assay sensitivity. The Bio-Rad CFX Opus 96 is considered representative of all the instruments validated for use with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit, as demonstrated during the LoD study shown previously and, therefore the confirmatory LoD study was performed only on this instrument. The LoD of each strain was determined separately using 20 independently extracted contrived samples prepared at various dilutions in negative clinical NP swab

matrix. See Table 20 for for the confirmatory LoD study data. The LoD for the contemporary influenza A and B strains was 589 and 593 copies/mL, respectively.

Table 20. LoD Summary for Contemporary Live Influenza Strains on the CFX Opus 96

Influenza A (A/Kansas/14/2017)		
Concentration Units		Positive Replicates/Total Replicates
copies/mL	$TCID_{50}$/mL	
1179	2.70	20/20
589	1.35	20/20
295	0.68	15/20
147	0.34	14/20
Influenza B (Colorado/06/2017 [Victoria])		
Concentration Units		Positive Replicates/Total Replicates
copies/mL	$TCID_{50}$/mL	
1186	8.8E-02	20/20
593	4.4E-02	20/20
296	2.2E-02	16/20
148	1.1E-02	6/20

LoD Using Inactivated Influenza Viruses

The co-infection and carryover studies were completed using inactivated viral organisms (Influenza A H1N1pdm and Influenza B Florida/02/06). In addition to the live cultured virus LoD study described above (for both contemporary and non-contemporary strains), the LoD of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT PCR Assay kit was also established using inactivated viral strains (Table 21) in clinical negative NP swab matrix on all four claimed instrument platforms.

LoD results (Table 22 and 23 and summarized in Table 24) demonstrate the limit of detection for inactivated influenza A virus is 500 to 1000 copies/mL and 250 to 500 copies/mL for inactivated influenza B virus. Each virus has equivalent LoD performance (within 2-3 fold) across all validated instruments with the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit.

Table 21. Stock Inactivated Viral Strains Used for LoD Studies

Virus	Strain	Source	Catalog No.	Stock Concentration
Influenza A	H1N1pdm	Zeptometrix	0810311CFNHI	2.26E07 copies/mL
Influenza B	Florida/02/06	Zeptometrix	NATFLUB-ST	4.12E09 copies/mL

Table 22. Limit of Detection of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit in Detecting Inactivated FluA Virus

FluA cp/mL	CFX96 Touch	CFX96 Dx	CFX Opus 96	AB7500 Fast Dx
	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates	FluA Positive replicates/Total replicates
1000	20/20	20/20	20/20	20/20
500	19/20	20/20	18/20	17/20
250	12/20	13/20	10/20	12/20
125	8/20	16/20	9/20	14/20

Table 23. Limit of Detection of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit in Detecting Inactivated FluB Virus

FluB cp/mL	CFX96 Touch	CFX96 Dx	CFX Opus 96	AB7500 Fast Dx
	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates	FluB Positive replicates/Total replicates
1000	20/20	20/20	20/20	20/20
500	20/20	20/20	20/20	20/20
250	20/20	20/20	18/20	18/20
125	17/20	17/20	10/20	18/20

Table 24. Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit LoD Summary for Inactivated FluA and FluB Viruses

Instrument	Inactive FluA	Inactive FluB
CFX96 Touch	500 copies/mL	250 copies/mL
CFX96 Dx	500 copies/mL	250 copies/mL
CFX Opus 96	1000 copies/mL	500 copies/mL
AB7500 Fast Dx	1000 copies/mL	500 copies/mL

Matrix Equivalency

A matrix equivalency study was performed to determine equivalent performance between contrived specimens prepared using clinical negative NP matrix and contrived specimens using simulated matrix. Contrived specimens in simulated matrix were utilized for the co-infection and carryover studies. To determine matrix equivalency, a selection of inactivated and live culture viruses from the LoD studies described previously were used to compare NP matrix to simulated matrix as listed in Table 25.

Table 25. Viral strains used for matrix equivalency study

Virus	Strain	LoD		Stock Concentration		3x LoD cp/mL	Vendor (P/N)
		cp/mL	$TCID_{50}$/mL	cp/mL	$TCID_{50}$/mL		
SARS-CoV-2	2019-nCoV/USA-WA1/2020	250	N/A	2.44E+08	N/A	750	ATCC (VR-1986HK)
Influenza A	H1N1 (A/PR/8/34)	1002	2.52	6.26E+09	1.58E+06	3006	ATCC (VR-1469)
	H3N2 (Kansas/14/17)	589	1.35	1.60E+08	3.89E+04	1767	Zeptometrix (0810586CF)

	H1N1pdm	1000	N/A	2.26E+07	N/A	3000	Zeptometrix (0810311CFNHI)
Influenza B	Lee/40	862	0.23	1.03E+10	2.81E+06	2586	ATCC (VR-1535)
	Colorado/06/2017 (Victoria)	593	0.044	4.60E+10	3.55E+05	1779	Zeptometrix (0810573CF)
	Florida/02/2006	500	N/A	4.12E+09	N/A	1500	Zeptometrix (NATFLUB-ST)

N/A; Inactivated strain, no $TCID_{50}$ concentration available

Three replicates per strain and per matrix type were created at 3-fold their respective LoD. The NP matrix samples were extracted using QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume). The nucleic acid from these extractions and the simulated matrix samples was tested with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on the CFX Opus 96. All controls performed as expected. Cq values at the testing concentration for each analyte were comparable between simulated and clinical matrix (i.e., ΔCq <1.5) with the exception of H3N2 which had a ΔCq of 3.4. The reason for this discrepancy is unclear; however, this strain of influenza A was not used in any additional studies with simulated matrix and the results were therefore deemed acceptable. The results are summarized in Table 26.

Table 26. Matrix equivalency study results

Virus	Strain	Clinical NP Matrix			Simulated Matrix			ΔCq
		Positive replicates	Avg Cq	StdDev	Positive replicates	Avg Cq	StdDev	
SARS-CoV-2	2019-nCoV/USA-WA1/2020	3/3	37.80	1.73	3/3	36.50	0.23	1.30
Influenza A	H3N2 (Kansas/14/17)	3/3	36.18	0.80	3/3	32.76	0.10	3.42
	H1N1 (A/PR/8/34)	3/3	38.82	0.67	3/3	37.48	0.72	1.34
	H1N1pdm	3/3	33.58	1.15	3/3	34.51	0.28	-0.93
Influenza B	Colorado/06/2017 (Victoria)	3/3	34.59	0.39	3/3	34.22	0.18	0.37
	Lee/40	3/3	36.18	2.49	3/3	37.42	0.43	-1.24
	Florida/02/2006	3/3	36.18	0.82	3/3	35.75	0.31	0.43

StdDev; Standard Deviation of Cq values; $\Delta Cq = Avg\ Cq_{Clinical} - Avg\ Cq_{Simulated}$

Inclusivity

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit contains primer/probe sets (FluA, FluB, and SC2) targeting RNA of influenza A, influenza B, and SARS-CoV-2 viruses. The oligonucleotide primers and probes for the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit are the same sequences as the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay (EUA received July 2, 2020).

Bio-Rad is using the CDC validated oligonucleotide primers and probe sequences by Right of Reference. The CDC inclusivity *in silico* analysis for SARS-CoV-2 as described in the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay IFU is referenced below.

SARS-CoV-2 *In Silico* Analysis:

CDC tested the inclusivity/exclusivity of each primer and probe oligonucleotide sequence for the SARS-CoV-2 target of the assay via NCBI BLAST+ against the nr/nt database, updated on 04/25/2020 (N=57791697 sequences analyzed), which confirmed only perfect matches to SARS-CoV-2 and close matches to SARS-CoV-2 ancestors (i.e., no genomes identified with more than 2 nt mismatches).

Additionally, Bio-Rad performed an alignment with the oligonucleotide primer and probe sequences of the SC2 assay with all publicly available nucleic acid sequences for SARS-CoV-2 in the Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org) and from the GenBank databases spanning from April 1, 2020 to November 15, 2020 to demonstrate the predicted inclusivity of the SC2 assay. An evaluation of 197,261 available SARS-CoV-2 sequences in GISAID and 38,738 available SARS-CoV-2 sequences in GenBank was done for this study (N=235,999 total sequences). This count includes both complete and partial genomes; only genomes with complete SC2 assay binding sites were analyzed. Results confirmed perfect and close matches to SARS-CoV-2 (no genomes with the SC2 binding site were identified to have more than 2 nucleotide mismatches in total across both primers and probe), as shown in Table 27a. When present, the mismatch could be found in any position of the assay binding site. The likelihood of one to two mismatches resulting in a significant loss in reactivity, and false negative result, is low. The assay and probe designs are optimized to be most efficient at annealing temperatures of 60°C, enabling the assay to withstand one or two mismatches.

Table 27a. *In silico* inclusivity analysis for SARS-CoV-2

Database	Genomes available[1]	Genomes with SC2 binding site[2]	Genomes with perfect match	Genomes with 1 mismatch	Inclusivity[3]
GISAID	197,261	196,382	187,730	8,285	99.81%
GenBank	38,738	38,442	36,911	1,512	99.95%
TOTAL	**235,999**	**234,824**	**224,641**	**9,797**	**99.84%**

[1]These numbers include both complete and partial genomes; some binding sites are missing due to incomplete genome or low-quality sequences
[2]When 'N' is found in the primer binding site, the target was considered to be missing information and excluded from inclusivity calculation
[3]Percentage of sequences with the SC2 binding site exhibiting ≤ 1 mismatch

In addition, a variant analysis was performed against the UK, South Africa, Brazil, and California strains (February 2021). Results confirmed perfect matches to the SARS-CoV-2 primers/probe. The predicted inclusivity of the SARS-CoV-2 assay is not impacted by the currently circulating escape variants.

Influenza *In Silico* Analysis:

An alignment with the oligonucleotide primer and probe sequences of the FluA and FluB assays with the publicly available nucleic acid sequences for laboratory and contemporary strains of influenza A and influenza B in the GenBank database was performed to demonstrate the predicted inclusivity of the FluA and FluB assays. For this analysis, an evaluation of 48 available influenza A sequences and 8 available influenza B sequences was performed. This count includes genomes with complete assay binding sites. Results confirmed that 48/48 influenza A genomes and 8/8 influenza B genomes had perfect and close matches to Influenza A and B respectively (no genomes with the FluA or FluB binding sites were identified to have more than 2 nucleotide mismatches), as shown in Table 27b. These mismatches occurred towards the middle and 5' end of the assays. The likelihood of one to two mismatches resulting in a significant loss in reactivity, and false negative result, is low. The assay and probe designs are optimized to be most efficient at annealing temperatures of 60°C, enabling the assay to withstand one or two mismatches.

Table 27b. *In silico* Inclusivity Analysis for Influenza A and B

Target	Genomes available[1]	Perfect Match	1 Mismatch	2 Mismatch	Inclusivity
FluA[2]	48	15	22	11	100%
FluB	8	5	3	0	100%

[1]These numbers include both complete and partial genomes, all of which have the complete binding sites

[2]FluA assay includes 2 pairs of forward and reverse primers. A mismatch was not included in the total count if one of the two primer pairs was a match at that same position.

Influenza Inclusivity (Wet Testing):

The inclusivity of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was evaluated using 13 influenza A and 9 influenza B viruses representing temporal, geographic, and genetic diversity within the subtype and lineage. All viral strains tested were quantified with Droplet Digital PCR to standardize concentration units and were prepared at two concentrations, 2x and 5x LoD (refer to Table 19 for Influenza A (H1N1 A/PR/8/34) and B (B/Lee/40) LoD concentrations). Samples were extracted in triplicate using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume) and were tested on the CFX Opus 96 with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. The influenza A target (FluA) generated positive results in both concentrations of the influenza A viruses tested. The influenza B target (FluB) generated positive results in both concentrations of the influenza B viruses tested. All controls performed as expected. Inclusivity evaluation results are presented below in Table 28.

Table 28. Inclusivity Evaluation – Influenza

Lineage	Strain Designation	Concentration		Extraction 1			Extraction 2			Extraction 3		
		EID_{50}/mL or PFU/mL*	cp/mL	SC2 FAM	FluA HEX	FluB Cy5	SC2FAM	FluA HEX	FluB Cy5	SC2 FAM	FluA HEX	FluB Cy5
A(H1N1)	A/Brisbane/02/2018	182	2004	N/A	34.85	N/A	N/A	35.16	N/A	N/A	35.21	N/A
		455	5010	N/A	33.52	N/A	N/A	34.03	N/A	N/A	33.56	N/A
A(H1N1)	A/Christ Church/16/2010	710	2004	N/A	34.91	N/A	N/A	34.74	N/A	N/A	34.81	N/A
		1775	5010	N/A	33.76	N/A	N/A	33.62	N/A	N/A	33.56	N/A
A(H3N2)	A/Kansas/14/2017	127	2004	N/A	34.66	N/A	N/A	34.32	N/A	N/A	34.40	N/A
		318	5010	N/A	32.83	N/A	N/A	32.71	N/A	N/A	32.95	N/A
A(H3N2)	A/Perth/16/2009	93.6	2004	N/A	34.79	N/A	N/A	34.84	N/A	N/A	35.08	N/A
		234	5010	N/A	33.16	N/A	N/A	33.61	N/A	N/A	33.87	N/A
A(H1N1)	A/PR/8/34	3.7	2004	N/A	36.05	N/A	N/A	36.47	N/A	N/A	36.08	N/A
		9.25	5010	N/A	34.45	N/A	N/A	34.96	N/A	N/A	34.87	N/A
A(H1N1)	A/WS/33	1.6	2004	N/A	37.24	N/A	N/A	38.06	N/A	N/A	37.29	N/A
		4	5010	N/A	36.57	N/A	N/A	35.91	N/A	N/A	36.18	N/A
A(H3N2)	A/Hong Kong/8/68	0.168	2004	N/A	36.86	N/A	N/A	36.50	N/A	N/A	36.85	N/A
		0.42	5010	N/A	35.44	N/A	N/A	35.56	N/A	N/A	35.34	N/A
A(H3N2)	A/Wisconsin/67/2005	341	2004	N/A	35.49	N/A	N/A	35.53	N/A	N/A	35.33	N/A
		853	5010	N/A	33.76	N/A	N/A	33.77	N/A	N/A	33.58	N/A
A(H1N1)	A1/Denver/1/57	198	2004	N/A	35.29	N/A	N/A	35.50	N/A	N/A	35.45	N/A
		495	5010	N/A	34.10	N/A	N/A	34.30	N/A	N/A	33.80	N/A
A(H3N2)	A/Port Chalmers/1/73	518	2004	N/A	34.97	N/A	N/A	35.11	N/A	N/A	34.98	N/A
		1295	5010	N/A	33.89	N/A	N/A	33.57	N/A	N/A	33.47	N/A
A(H3N2)	A/Victoria/3/75	11.3	2004	N/A	36.21	N/A	N/A	35.83	N/A	N/A	35.66	N/A
		28.3	5010	N/A	35.06	N/A	N/A	34.26	N/A	N/A	34.19	N/A
A(H1N1)	A/New Jersey/8/76	42.8	2004	N/A	36.50	N/A	N/A	36.25	N/A	N/A	36.59	N/A
		107	5010	N/A	34.62	N/A	N/A	34.68	N/A	N/A	34.67	N/A
A(H1N1)	A/FM/1/47	394	2004	N/A	33.52	N/A	N/A	33.52	N/A	N/A	33.29	N/A
		985	5010	N/A	32.31	N/A	N/A	32.24	N/A	N/A	32.11	N/A
B (Victoria)	B/Colorado/06/2017	13.8	1724	N/A	N/A	34.23	N/A	N/A	34.56	N/A	N/A	34.62
		34.5	4310	N/A	N/A	32.73	N/A	N/A	33.11	N/A	N/A	33.45
B (Victoria)	B/Michigan/09/2011	33.3	1724	N/A	N/A	35.12	N/A	N/A	36.45	N/A	N/A	36.05
		83.3	4310	N/A	N/A	34.31	N/A	N/A	34.51	N/A	N/A	34.24
B		240	1724	N/A	N/A	35.51	N/A	N/A	36.13	N/A	N/A	36.03

Lineage	Strain Designation	Concentration		Extraction 1			Extraction 2			Extraction 3		
		EID_{50}/mL or PFU/mL*	cp/mL	SC2 FAM	FluA HEX	FluB Cy5	SC2FAM	FluA HEX	FluB Cy5	SC2 FAM	FluA HEX	FluB Cy5
(Yamagata)	B/New Hampshire/01/2016	600	4310	N/A	N/A	34.62	N/A	N/A	34.52	N/A	N/A	34.72
B (Yamagata)	B/Phuket/3073/2013	119	1724	N/A	N/A	34.43	N/A	N/A	33.81	N/A	N/A	32.82
		297	4310	N/A	N/A	32.55	N/A	N/A	32.90	N/A	N/A	31.44
B (Yamagata)	B/Florida/2006	1.4	1724	N/A	N/A	35.01	N/A	N/A	35.15	N/A	N/A	35.21
		3.5	4310	N/A	N/A	34.00	N/A	N/A	34.18	N/A	N/A	34.38
B	B/Lee/41	0.43	1724	N/A	N/A	35.08	N/A	N/A	34.28	N/A	N/A	35.05
		1.08	4310	N/A	N/A	33.62	N/A	N/A	33.60	N/A	N/A	34.02
B	B/Allen/45	2.74	1724	N/A	N/A	35.96	N/A	N/A	35.97	N/A	N/A	36.13
		6.85	4310	N/A	N/A	34.22	N/A	N/A	34.32	N/A	N/A	34.72
B	B/GL/1739/54	64.6	1724	N/A	N/A	34.82	N/A	N/A	34.88	N/A	N/A	34.15
		162	4310	N/A	N/A	32.97	N/A	N/A	33.26	N/A	N/A	32.61
B	B/Maryland/1/59	0.49	1724	N/A	N/A	35.11	N/A	N/A	35.28	N/A	N/A	34.90
		1.23	4310	N/A	N/A	33.86	N/A	N/A	34.12	N/A	N/A	33.47

* - Influenza A strains: Brisbane, Christ Church, Kansas and Perth concentrations reported in EID50/mL
- Influenza B strains: Colorado, Michigan, New Hampshire and Phuket concentrations reported in EID50/mL
- Influenza A strains: A/PR/8/34 and A/WS33 concentrations reported in PFU/mL
- All other Influenza A and B strains concentrations reported in $CEID_{50}$/mL

LoD on the CFX Opus 96 for influenza A and influenza B is 1002 copies/mL and 862 copies/mL; the two concentrations displayed in copies/mL represent 2X and 5X LoD.
N/A; Negative, no detectable Cq value

CDC Human Influenza Virus Panel (2019):

The inclusivity of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was also evaluated with the CDC Human Influenza Virus Panel (2019), which is comprised 8 contemporary influenza A and B strains, 4 influenza A viruses, and 4 influenza B viruses (Table 29) per the protocol provided with the panel. For each stock virus, a five-fold dilution series was prepared in saline. Five replicates per dilution were extracted using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume) and tested on the CFX Opus 96 with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. Testing the dilution series was performed until there was non-reactivity at two consecutive five-fold dilution levels, as shown by obtaining zero positive results for all five replicates. The last dilution that produced positive results in at least one out of the five replicates tested was considered the minimum reactive concentration and is shown in Table 29. All controls performed as expected.

Table 29. CDC Human Influenza 2019 Panel Members

Type	Subtype	Influenza Viral Strain	Stock Concentration (EID_{50}/mL)	Minimum Reactive Concentration (EID_{50}/mL)	Minimum Reactive Concentration (copies/mL)
A	H3N2	A/Perth/16/2009	$10^{8.3}$	2.04E+01	4.39E+02
A	H3N2	A/Kansas/14/2017	$10^{8.9}$	8.13E+01	1.27E+03
A	H1N1	A/Christ Church/16/2010	$10^{9.9}$	1.63E+02	4.57E+02
A	H1N1	A/Brisbane/02/2018	$10^{7.9}$	8.13E+00	8.92E+02
B	Victoria	B/Michigan/09/2011	$10^{8.5}$	2.60E-01	1.36E+01
B	Victoria	B/Colorado/06/2017	$10^{8.3}$	4.00E+00	5.12E+02
B	Yamagata	B/New Hampshire/01/2016	$10^{8.9}$	4.07E+02	2.90E+03
B	Yamagata	B/Phuket/3073/2013	$10^{8.9}$	8.13E+01	1.18E+03

Analytical Specificity (Cross-Reactivity/Microbial Interference)

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit contains primer/probe sets (FluA, FluB, and SC2) targeting RNA of influenza A, influenza B, and SARS-CoV-2 viruses. The oligonucleotide primers and probes for the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit are the same sequences as the CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay (EUA received July 2, 2020). Specifically, the influenza A and influenza B primers/probes are identical to those used in the 510(k) cleared CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K080570 and associated special 510(k) submissions). Bio-Rad is using the CDC validated oligonucleotide primers and probe sequences by Right of Reference. Therefore, additional *in silico* cross-reactivity analysis with the influenza A and B primers and probes was not performed, and only laboratory testing to verify the analytical specificity of the Reliance assay was conducted.

In silico Analysis

An *in silico* analysis was performed to assess the potential for "off-panel" organisms to interfere with SARS-CoV-2 detection. No significant homology was observed between the SARS-CoV-2 primers and probe and other coronaviruses or human microflora that is predicted to lead to false results with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit.

Wet Testing (Absence of On-Panel Organisms)

The cross-reactivity of each primer/probe set to viruses targeted by another component of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was evaluated by testing the influenza viruses from the inclusivity study (Table 30). Additionally, the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was evaluated for cross-reactivity by testing other viruses and pathogens including those commonly found in respiratory tract (Tables 31 and 32).

All pathogens (non-influenza viruses, bacteria, and fungi) were extracted in triplicate using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume). Nucleic acid from high titer preparations of the 64 organisms listed in Table 30 (22 influenza viruses), Table 31 (24 non-influenza viruses), and Table 32 (19 bacteria and fungi) representing respiratory pathogens or flora commonly present in human respiratory specimens and genetic near-neighbors of viruses targeted by the assay were tested in triplicate on the CFX Opus 96 with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. All controls performed as expected. The results are presented below in Table 30, Table 31, and Table 32.

Table 30. Exclusivity Evaluation – Influenza

Lineage	Strain Designation	Conc.*	Conc. cp/mL	Cq								
				Extraction 1			Extraction 2			Extraction 3		
				SC2 FAM	FluA HEX	FluB Cy5	SC2 FAM	FluA HEX	FluB Cy5	SC2 FAM	FluA HEX	FluB Cy5
A(H1N1)	A/Brisbane/02/2018	1.00E+06	1.10E+07	N/A	19.5	N/A	N/A	19.34	N/A	N/A	19.32	N/A
A(H1N1)	A/Christ Church/16/2010	1.00E+08	2.82E+08	N/A	18.04	N/A	N/A	18.29	N/A	N/A	18.46	N/A
A(H3N2)	A/Kansas/14/2017	1.00E+07	1.57E+08	N/A	18.34	N/A	N/A	18.56	N/A	N/A	18.44	N/A
A(H3N2)	A/Perth/16/2009	1.00E+06	2.14E+07	N/A	19.54	N/A	N/A	19.45	N/A	N/A	19.59	N/A
A(H1N1)	A/PR/8/34	6.70E+06	3.63E+07	N/A	14.5	N/A	N/A	14.9	N/A	N/A	14.94	N/A
A(H1N1)	A/WS/33	7.30E+06	9.15E+09	N/A	13.42	N/A	N/A	13.75	N/A	N/A	13.63	N/A
A(H3N2)	A/Hong Kong/8/68	1.60E+06	3.57E+08	N/A	17.41	N/A	N/A	17.47	N/A	N/A	17.56	N/A
A(H3N2)	A/Wisconsin/67/2005	2.30E+07	1.35E+08	N/A	18.02	N/A	N/A	18.09	N/A	N/A	18.08	N/A
A(H1N1)	A1/Denver/1/57	3.40E+07	3.43E+08	N/A	18.5	N/A	N/A	18.48	N/A	N/A	18.38	N/A
A(H3N2)	A/Port Chalmers/1/73	2.50E+07	9.69E+07	N/A	18.63	N/A	N/A	18.29	N/A	N/A	18.58	N/A
A(H3N2)	A/Victoria/3/75	1.60E+06	2.85E+08	N/A	17.34	N/A	N/A	17.44	N/A	N/A	17.95	N/A
A(H1N1)	A/New Jersey/8/76	5.00E+05	2.34E+07	N/A	14.04	N/A	N/A	14.1	N/A	N/A	14.06	N/A
A(H1N1)	A/FM/1/47	5.00E+06	2.54E+07	N/A	20.28	N/A	N/A	20.05	N/A	N/A	20.19	N/A
Influenza B	B/Colorado/06/2017	1.00E+06	1.25E+08	N/A	N/A	18.1	N/A	N/A	18.35	N/A	N/A	18.07
Influenza B	B/Michigan/09/2011	1.00E+07	5.18E+08	N/A	N/A	18.3	N/A	N/A	18.18	N/A	N/A	18.09
Influenza B	B/New Hampshire/01/2016	1.00E+06	7.16E+06	N/A	N/A	20.69	N/A	N/A	20.36	N/A	N/A	20.3
Influenza B	B/Phuket/3073/2013	1.00E+07	1.45E+08	N/A	N/A	18.94	N/A	N/A	18.38	N/A	N/A	18.43
Influenza B	B/Lee/41	1.60E+06	1.97E+09	N/A	N/A	14.99	N/A	N/A	15.38	N/A	N/A	15.33
Influenza B	B/Allen/45	1.20E+05	4.82E+08	N/A	N/A	17	N/A	N/A	17.59	N/A	N/A	17.17
Influenza B	B/GL/1739/54	5.00E+06	3.14E+09	N/A	N/A	14.71	N/A	N/A	14.67	N/A	N/A	14.53
Influenza B	B/Florida/2006	3.90E+08	1.04E+09	N/A	N/A	22.43	N/A	N/A	22.47	N/A	N/A	22.33
Influenza B	B/Maryland/1/59	6.80E+05	2.38E+09	N/A	N/A	14.85	N/A	N/A	23.36	N/A	N/A	14.34

*Influenza A strains: Brisbane, Christ Church, Kansas and Perth concentrations reported in EID_{50}/mL
*Influenza B strains: Colorado, Michigan, New Hampshire and Phuket concentrations reported in EID_{50}/mL
*Influenza A strains: A/PR/8/34 and A/WS33 concentrations reported in PFU/mL
*All other influenza A and B strains concentrations reported in $CEID_{50}$/mL
N/A; Negative, no detectable Cq value

Table 31. Exclusivity Evaluation – Non-Influenza Viruses

Lineage	Strain Designation	Concentration PFU/mL	Bio-Rad Reliance SARS-CoV-2/FluA/FluB Assay Cq								
			Extraction 1			Extraction 2			Extraction 3		
			SC2	FluA	FluB	SC2	FluA	FluB	SC2	FluA	FluB
Adenovirus	3	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	31	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	4	1.11E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	40/Dugan	8.82E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	41/TAK	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	5	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	7A	1.11E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Adenovirus	1	1.54E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Betacoronavirus 1	OC43	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Enterovirus	68	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Epstein-Barr	B95-8	3.50E+03	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Human coronavirus	HCoV HKU-1	5.00E+04*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parainfluenza 1	C35	5.00E+04*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Human Rhinovirus		3.51E+03	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Lineage	Strain Designation	Concentration PFU/mL	Bio-Rad Reliance SARS-CoV-2/FluA/FluB Assay Cq								
			Extraction 1			Extraction 2			Extraction 3		
			SC2	FluA	FluB	SC2	FluA	FluB	SC2	FluA	FluB
Herpes Simplex, 1	MacIntyre	1.96E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Varicella-Zoster	Ellen	1.96E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Measles	Edmonston	6.23E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mumps	Enders	1.96E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cytomegalorvirus	AD-169	1.64E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Human metapneumovirus		2.72E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parainfluenza 2		1.12E+05*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parainfluenza 3		1.12E+05*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parechovirus A	Type 3	1.12E+05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Human respiratory syncytial virus	A2	5.00E+04*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

*Human coronavirus, Parainfluenza 1, and Human respiratory syncytial virus concentrations in copies/mL
N/A; Negative, no detectable Cq value

Table 32. Exclusivity Evaluation – Bacteria and Fungi

Lineage (Strain Designation)	Concentration CFU/mL	Bio-Rad Reliance SARS-CoV-2/FluA/FluB Assay Cq								
		Extraction 1			Extraction 2			Extraction 3		
		SC2	FluA	FluB	SC2	FluA	FluB	SC2	FluA	FluB
Bordetella pertussis	5.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cornyebacterium striatum	2.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Escherichia coli (H10407)	1.06E+07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Haemophilus influenza (KW20)	2.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Legionella anisa	1.40E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lactobacillus planatarum	3.00E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Moraxella catarrhalis	5.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mycoplasma; pneumoniae (M129-B7)	3.00E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mycobacerium tuberculosis	1.40E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Neisseria gonorrhoeae	5.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Neisseria meningitidis	2.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pseudomonas aeruginosa (Boston 41501)	6.64E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Staphylococcus aureus, subsp. aureus	1.60E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Staphylococcus epidermis	3.00E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Staphylococcus pneumoniae (TIGR4)	3.00E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Streptococcus pyogenes	3.60E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Streptococcus salivarius	1.40E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Candida albicans	2.09E+06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pneumocystis jiroveci	5.00E+04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

**Bordetella pertussis, Moraxella catarrhalis and Pneumocystis jiroveci* concentrations in copies/mL
N/A; Negative, no detectable Cq value

Wet Testing (Presence of On-Panel Influenza Organisms) (Microbial Interference)

The potential for cross-reaction and/or microbial interference was further assessed by testing off-panel virsues, bacteria and fungi in the presence of a low level of influenza viruses as representative analytes. The interfering pathogens were tested at the highest concentration possible (e.g. diluted ≥10^6 CFU/mL for bacteria, ≥10^5 PFU/mL for viruses, undiluted when pathogen stock concentration was lower) and were mixed with an influenza virus at 2x LoD concentration in the same sample to determine if the presence of the non-target organism would interfere with the detection of the influenza analyte using the Bio-Rad

Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. Refer to Table 20 for Influenza A and B LoD concentrations of these contemporary strains.

Contrived samples were created with one contemporary virus strain from each influenza type: Influenza A (H3N2) A/Kansas/14/2017 at 2x LoD (1178 cp/mL) and Influenza B (B/Colorado/06/2017) at 2x LoD (1186 cp/mL). The influenza viruses and non-influenza organisms were spiked into a clinical negative NP swab matrix pool at the desired concentrations. Each contrived sample was extracted using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume) in triplicate. The nucleic acid from these extractions was tested with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on the CFX Opus 96 instrument. All controls performed as expected. No microbial interference was observed with the pathogens tested. All samples were positive in 3/3 replicates for influenza A or influenza B.

Interfering Substances Study

The potential interference of substances commonly found in nasal swab samples on the sensitivity of viral detection by the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was assessed in an interfering substance study. This study utilized contemporary influenza A and influenza B strains, the LoD for which are contained in Table 33. Refer to the LoD section for additional information.

Table 33. Summary of LoD of Viral Strains Evaluated in the Interfering Substances Study

Virus	Strain	LoD (CFX Opus 96)	3x LoD (CFX Opus 96)
SARS-CoV-2	2019-nCoV/USA-WA1/2020	250 cp/mL	750 cp/mL
Influenza A	H3N2 (Kansas/14/17)	589 cp/mL	1,767 cp/mL
Influenza B	Colorado/06/2017 (Victoria)	593 cp/mL	1,779 cp/mL

The interfering substance study utilized contrived samples comprised of contemporary influenza viral strains from live influenza A, influenza B, or inactivated SARS-CoV-2 virus in a negative nasopharyngeal swab matrix. Contrived samples were prepared by spiking each virus individually at 3x LoD into a clinical negative matrix pool; a no virus control sample was also prepared. Potentially interfering substances were added to the contrived samples at concentrations that represent the highest levels expected in human respiratory patient samples based on a review of the literature as well as decision summaries of 510(k) cleared influenza assays. A negative control that used water as the added substance was also included. Each sample was extracted in triplicate using the QIAamp Viral RNA Mini Kit and tested as directed in the IFU on the CFX Opus 96 instrument.

This study demonstrates these substances, except for FluMist Quadrivalent (2020-2021 Formula, MedImmune/AstraZeneca), do not interfere with SARS-CoV-2, influenza A, or influenza B virus detection. All contrived samples tested positive as expected in the presence of the interfering substance and tested negative in the no virus control sample (Table 34). FluMist Quadrivalent contains weakened influenza A and influenza B virus and, as expected, is positive for these viruses in the no virus control sample screened with the FluA and FluB assays. FluMist Quadrivalent does not impact the sensitivity to detect the SARS-CoV-2 virus (Table 34). These data indicate the interfering substances at the high concentrations tested do not impact virus detection sensitivity of the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit.

Note: Individuals who received nasally administered influenza A/B vaccine may have positive influenza test results for up to three days after vaccination.

Table 34. Interfering Substance Study Results

Substance	Active Agent	Conc. Tested	SC2 Assay			FluA Assay			FluB Assay		
			# Positive out of 3 replicates		SC2 Mean Cq[1]	# Positive out of 3 replicates		FluA Mean Cq[1]	# Positive out of 3 replicates		FluB Mean Cq[1]
			SC2 virus	No virus		FluA virus	No virus		FluB virus	No virus	
Mucin	Purified mucin protein	40 mg/mL	3	0	37.66	3	0	37.72	3	0	37.39
Nasal Cortico-steroids	Dexamethasone	3 mg/mL	3	0	36.04	3	0	35.46	3	0	36.65
	Fluticasone	2 mg/mL	3	0	37.12	3	0	35.95	3	0	36.09
	Mometasone Furoate	1 mg/mL	3	0	35.97	3	0	35.06	3	0	35.49
	Budesonide	1 mg/mL	3	0	36.07	3	0	35.49	3	0	36.17
	Flunisolide	5 mg/mL	3	0	35.84	3	0	35.84	3	0	36.19
	Triamcinolone acetonide	1.5 mg/mL	3	0	36.17	3	0	35.50	3	0	36.13
	Beclomethasone	2 mg/mL	3	0	35.85	3	0	35.90	3	0	36.41
Throat Lozenges	Benzocaine	7.5 mg/mL	3	0	37.05	3	0	36.39	3	0	36.43
	Menthol	7.5 mg/mL	3	0	38.30	3	0	36.32	3	0	36.14
	Zinc Acetate	7.5 mg/mL	3	0	37.43	3	0	36.17	3	0	36.25
	Chloraseptic Max Strength Sore Throat Lozenges (contains Benzocaine and Menthol)	10 mg/mL	3	0	37.75	3	0	35.89	3	0	36.98
Nasal Sprays or Drops	Oxymetazoline	10 mg/mL	3	0	38.42	3	0	36.13	3	0	36.47
	Phenylephrine	0.5 mg/mL	3	0	38.40	3	0	36.26	3	0	35.97
	Sodium chloride w/ preservatives	0.2 mg/mL	3	0	35.47	3	0	35.65	3	0	35.78
Mupirocin	Mupirocin	2 mg/mL	3	0	38.92	3	0	36.23	3	0	36.39
Tobramycin	Tobramycin	2 mg/mL	3	0	37.47	3	0	36.00	3	0	36.17
Zanamivir	Zanamivir	5 mg/mL	3	0	38.59	3	0	36.25	3	0	36.45
Allergy Medicine	Histaminum hydrochloricum	20% v/v	3	0	36.75	3	0	35.55	3	0	36.29
	Triple allergy defense (contains Galphimia glauca, Histaminum hydrochloricum, Luffa operculata, Sabadilla)	20% v/v	3	0	35.37	3	0	35.79	3	0	36.53
	Sulphur 12X	10 mg/mL	3	0	35.85	3	0	35.47	3	0	35.88
FluMist Quadrivalent	Weakened FluA/B virus	20% v/v	3	0	35.83	3	3	16.21	3	3	15.66
Blood	N/A	5% v/v	3	0	35.41	3	0	35.96	3	0	36.21
Water (control)	N/A	10% v/v	3	0	37.74	3	0	35.56	3	0	36.21

N/A; Not Applicable

[1] Mean Cq for samples containing virus

Carryover/Cross-Contamination Study

The effects of carryover or cross-contamination were tested on the CFX Opus 96 and CFX96 Touch. Contrived high concentration viral RNA samples were plated adjacent to negative samples in an alternating pattern and tested using the Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. For this study, high concentration SC2 (Italy-IMNI1, Zeptometrix, Cat# 0810589CFHI1) = 2,000,000 cp/mL; FluA (H1N1pdm, Zeptometrix, Cat#0810311CFNHI) = 2,575,000 cp/mL; FluB (B/Lee/40, Exact Diagnostics, Cat# 130062) = 2,850,000 cp/mL. The negative samples were prepared using total human RNA (Thermo Fisher, Cat. #AM7852) to simulate negative human specimens. Multiple runs were performed allowing for the detection of any run-to-run cross-contamination. There was no high concentration carryover or cross-contamination from well-to-well or run-to-run.

Coinfection (Competitive Interference)

A coinfection study was performed to determine if the sensitivity of detecting a particular virus is affected when high levels of the other two viruses are present. Contrived samples were prepared using simulated matrix and the strains in Table 35. The co-infection combinations that were evaluated in this study are shown in Table 36. High concentration is defined as follows: SC2 = 1,381,300 cp/mL; FluA = 2,575,000 cp/mL; FluB = 2,850,000 cp/mL. Each test virus dilution point was analyzed in triplicate, using Bio-Rad's Reliance SARS-CoV-2/FluA/FluB RT-PCR Test Kit, to identify the lowest amount of virus RNA that can be detected in all three replicates. This test was performed on the CFX Opus 96 instrument. The NTC, positive, and negative controls were included in each plate; the results were as expected.

Table 35. Virus Selected for Coinfection Study

Virus	Type	Strain	Company	Catalog #	Stock Titer (cp/mL)
SARS-CoV-2	N/A	Italy INMI1	Zeptometrix	0810589CFHI	2.28E07
Influenza	A	H1N1pdm	Zeptometrix	0810311CFNHI	4.12E09
Influenza	B	B/Lee/40	Exact Diagnostics	130062	2.55E10

Table 36. Combinations Tested for Coinfection Study

Virus	Titer		
SARS-CoV-2	High	High	Serial dilution
Influenza A	High	Serial dilution	High
Influenza B	Serial dilution	High	High

Refer to Table 37 for the results of the study. The results demonstrate that when using Bio-Rad's Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit, there is no observed loss in the sensitivity of detecting a test virus in samples that have high levels of other viruses present.

Table 37. Coinfection Results using the CFX Opus 96 Instrument

Test Virus	Test virus RNA copies/mL	SC2 assay				FluA assay				FluB assay			
		SC2 Only		SC2 with High Titer FluA/FluB		FluA Only		FluA with High Titer SC2/FluB		FluB Only		FluB with High Titer SC2/FluA	
		# Pos	Avg Cq	# Pos	Avg Cq	# Pos	Avg Cq	# Pos	Avg Cq	# Pos	Avg Cq	# Pos	Avg Cq
SC2	2000	3/3	34.50	3/3	34.45	0/3	N/A	3/3	24.71	0/3	N/A	3/3	23.97
	1000	3/3	35.65	3/3	35.56	0/3	N/A	3/3	24.70	0/3	N/A	3/3	24.05
	500	3/3	36.35	3/3	36.37	0/3	N/A	3/3	24.77	0/3	N/A	3/3	24.04
	250	3/3	37.66	3/3	37.81	0/3	N/A	3/3	24.66	0/3	N/A	3/3	24.01
	125	1/3	38.28	1/3	39.37	1/3*	39.98	3/3	24.73	0/3	N/A	3/3	24.04
	62.5	1/3	39.37	2/3	38.73	0/3	N/A	3/3	24.74	0/3	N/A	3/3	24.09
	0	0/3	N/A	0/3	N/A	0/3	N/A	3/3	24.66	0/3	N/A	3/3	23.98
FluA	2000	0/3	N/A	3/3	24.69	3/3	35.55	3/3	35.13	0/3	N/A	3/3	24.06
	1000	0/3	N/A	3/3	24.65	3/3	36.27	3/3	37.08	0/3	N/A	3/3	24.09
	500	0/3	N/A	3/3	24.67	3/3	38.27	3/3	37.77	0/3	N/A	3/3	24.06
	250	0/3	N/A	3/3	24.65	2/3	38.99	2/3	39.38	0/3	N/A	3/3	24.09
	125	0/3	N/A	3/3	24.60	2/3	38.32	1/3	39.83	0/3	N/A	3/3	24.02
	62.5	0/3	N/A	3/3	24.57	1/3	38.61	2/3	39.91	0/3	N/A	3/3	24.07
	0	0/3	N/A	3/3	24.61	0/3	N/A	0/3	N/A	0/3	N/A	3/3	24.05
FluB	2000	0/3	N/A	3/3	24.76	0/3	N/A	3/3	24.86	3/3	35.09	3/3	35.03
	1000	0/3	N/A	3/3	24.77	0/3	N/A	3/3	24.82	3/3	36.02	3/3	36.21
	500	0/3	N/A	3/3	24.72	0/3	N/A	3/3	24.83	3/3	37.09	3/3	36.78
	250	0/3	N/A	3/3	24.74	0/3	N/A	3/3	24.81	3/3	37.82	3/3	38.30
	125	0/3	N/A	3/3	24.63	0/3	N/A	3/3	24.74	1/3	37.89	2/3	38.65
	62.5	0/3	N/A	3/3	24.72	0/3	N/A	3/3	24.82	0/3	40.11	0/3	N/A
	0	0/3	N/A	3/3	24.68	0/3	N/A	3/3	24.78	0/3	N/A	0/3	N/A

N/A; Negative, no detectable Cq value

*One of the three SARS-CoV-2 replicates at 125 copies/mL showed a delayed Cq of 39.98 for FluA. Although considered a positive FluA result, this positive replicate is due to trace contamination levels while preparing and processing the high-titer contrived samples for this study and considered unrelated to cross-reactivity of the assays.

Precision/Repeatability

The within-laboratory precision of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was established using two reagent lots and two instruments, the CFX Opus 96 and the CFX96 Touch RT-PCR Systems. The precision panel consisted of negative samples as well as individually spiked live cultured influenza A and influenza B viruses that were used in the LoD studies (H1N1 A/PR/8/34, ATCC, Cat# VR-1469; Lee/40, ATCC, Cat# VR-1535). Samples were prepared at low and moderate positive concentrations (2X LoD and 5X LoD, respectively) in pooled SARS-CoV-2/influenza A/influenza B negative clinical NP swab matrix (Tabel 38). For each data point, all panel members were extracted five times using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume). Nucleic acid from these extractions was tested on the CFX Opus 96 and the CFX96 Touch with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. One operator tested the 5-member reproducibility panel with 5 replicates on 3 different days on 2 different instruments for a total of 300 replicates (5 panel members x 5 replicates x 2 lots x 3 days x 2 instruments).

The expected result for the negative panel member was “Not Detected,” while the expected result for the low and moderate positive panel members was “Detected” for the respective target. The data demonstrate an acceptable level of precision for the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit. Detailed results are summarized in Table 39 and Table 40 for Influenza A and Influenza B, respectively. The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit exhibited the expected hit rates for all targets.

Table 38. Concentration of Panel Members and Correlation to LoD Per Instrument

Panel Member	Concentration (copies/mL)	Correlation to Live Influenza LoD	
		CFX Opus 96	CFX96 Touch
Influenza A Low +	2004	2X	2X
Influenza A Med +	5010	5X	5X
Influenza B Low +	1724	2X	2X
Influenza B Med +	4310	5X	5X
Negative	N/A	N/A	N/A

N/A; Negative, no detectable Cq value

Table 39. Influenza A Precision – Summary of Detection Rates

Panel Member	CFX Opus 96			CFX96 Touch			Total	
	Agreement w/Expected Result	Mean Cq	Cq %CV	Agreement w/Expected Result	Mean Cq	Cq %CV	Agreement w/Expected Result	Cq [95% CI]
Negative	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
FluA mod+	30/30	33.81	0.72	30/30	34.00	0.84	60/60	[93.98, 100]
FluA low+	30/30	35.79	1.75	30/30	36.06	1.32	60/60	[93.98, 100]
FluB mod+	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
FluB low+	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
Total Agreement	**150/150**			**150/150**			**300/300**	

N/A; Negative, no detectable Cq value

Table 40. Influenza B Precision – Summary of Detection Rates

Panel Member	CFX Opus 96			CFX96 Touch			Total	
	Agreement w/Expected Result	Mean Cq	Cq %CV	Agreement w/Expected Result	Mean Cq	Cq %CV	Agreement w/Expected Result	Cq [95% CI]
Negative	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
FluA mod+	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
FluA low+	30/30	N/A	N/A	30/30	N/A	N/A	60/60	N/A
FluB mod+	30/30	35.91	1.60	30/30	35.64	1.51	60/60	[93.98, 100]
FluB low+	30/30	37.13	1.55	30/30	37.00	1.45	60/60	[93.98, 100]
Total Agreement	**150/150**			**150/150**			**300/300**	

N/A; Negative, no detectable Cq value

The variance component analysis for each level and target is summarized in Table 41 and Table 42.

Table 41. Precision/Repeatability Study (Within-Run) - Overall Mean, Standard Deviations (SD), and Coefficients of Variation (CV%) for Target Cq Values

Run	FluA Mod +			FluA Low +			FluB Mod+			FluB Low+			Negative		
	Mean Cq	SD	%CV	Mean Cq	SD	%CV	Mean Cq	SD	%CV	Mean Cq	SD	%CV	Mean Cq	SD	%CV
CFX Opus 96															
Run 1	33.71	0.25	0.74	35.84	0.38	1.06	36.20	0.46	1.27	37.27	0.71	1.92	N/A	N/A	N/A
Run 2	33.79	0.21	0.63	35.24	0.44	1.26	35.40	0.35	0.98	37.02	0.55	1.48	N/A	N/A	N/A
Run 3	33.97	0.24	0.70	36.30	0.54	1.48	36.12	0.22	0.60	37.10	0.47	1.26	N/A	N/A	N/A
CFX96 Touch															
Run 1	33.80	0.16	0.47	35.84	0.36	1.00	35.53	0.36	1.02	36.83	0.64	1.75	N/A	N/A	N/A
Run 2	33.97	0.29	0.85	36.08	0.44	1.21	35.56	0.54	1.53	37.11	0.50	1.34	N/A	N/A	N/A
Run 3	34.23	0.23	0.67	36.25	0.56	1.53	35.82	0.44	1.22	37.07	0.47	1.25	N/A	N/A	N/A

N/A; Negative, no detectable Cq value

Table 42. Precision/Repeatability Study (Lot-to-Lot, Day-to-Day, and Run-to-Run) - Overall Mean, Standard Deviations (SD), and Coefficients of Variation (CV%) for Target Cq Values

Target	Panel Member	Detection Rate	Mean Cq	Lot-to-Lot		Day-to-Day		Run-to-Run	
				SD	CV%	SD	CV%	SD	CV%
CFX Opus 96									
FluA	Mod +	100%	33.81	0.24	0.72	0.24	0.72	0.24	0.72
	Low +	100%	35.79	0.63	1.75	0.63	1.75	0.63	1.75
	Negative	100%	N/A	N/A	N/A	N/A	N/A	N/A	N/A
FluB	Mod +	100%	35.91	0.50	1.39	0.57	1.60	0.57	1.60
	Low +	100%	37.13	0.57	1.55	0.57	1.55	0.57	1.55
	Negative	100%	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CFX96 Touch									
FluA	Mod +	100%	34.00	0.29	0.84	0.29	0.84	0.29	0.84
	Low +	100%	36.06	0.48	1.32	0.48	1.32	0.48	1.32
	Negative	100%	N/A	N/A	N/A	N/A	N/A	N/A	N/A
FluB	Mod +	100%	35.64	0.46	1.28	0.54	1.51	0.54	1.51
	Low +	100%	37.00	0.54	1.45	0.54	1.45	0.54	1.45
	Negative	100%	N/A	N/A	N/A	N/A	N/A	N/A	N/A

N/A; Negative, no detectable Cq value

Fresh vs Frozen

The Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit was tested by comparing performance of fresh specimens compared to the same specimens after repeated freeze/thaw cycles. One influenza A strain (Influenza A H3N2 (Kansas/14/17), ZeptoMetrix, Cat# 0810586CF), one influenza B strain (Influenza B (Colorado/06/17), ZeptoMetrix, Cat# 0810573CF), and one SARS-CoV-2 strain (2019-nCoV/USA-WA1/2020, ATCC VR-1986HK) were individually spiked into negative clinical NP swab matrix received within 72 hours of sample collection (shipped unfrozen with cold packs), at two different viral concentrations reflecting 2x and 5x LoD for each virus, as described in Table 43. A set of negative clinical samples was also tested.

For each viral strain, 20 fresh samples (10 per concentration per virus) were extracted using the QIAGEN QIAamp Viral RNA Mini Kit (140 µL sample input and 60 µL elution volume), and the nucleic acid from these extractions was tested with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit on one CFX Opus

96 instrument. A second set of 20 samples (10 per concentration per virus) underwent 3 freeze/thaw cycles (frozen at -80°C then thawed at room temperature), extracted, and tested with the same kit and instrument. All controls performed as expected. Agreement in detection between fresh and frozen samples was 100% for the two concentrations for influenza A, influenza B, and SARS-CoV-2, as well as for the negative samples for all freeze/thaw cycles. There was no evidence of a trend in Cq values that would indicate a difference in performance for any of the analytes that were present in contrived samples that underwent up to three sequential freeze/thaw cyclesResults are shown in Table 44.

Table 43. Contemporary Viral Strains and Concentrations Tested in the Fresh vs. Frozen Study

Virus	Strain	LoD	2X LoD	5X LoD
SARS-CoV-2	2019-nCoV/USA-WA1/2020	250 copies/mL	500 copies/mL	1250 copies/mL
Influenza A	H3N2 (Kansas/14/17)	589 copies/mL	1178 copies/mL	2945 copies/mL
Influenza B	Colorado/06/2017	593 copies/mL	1186 copies/mL	2965 copies/mL

Table 44. Fresh versus Frozen Study Results

Sample Type		Fresh			Freeze/Thaw Cycle 1			Freeze/Thaw Cycle 2			Freeze/Thaw Cycle 3			Total Positivity
		SC2	FluA	FluB	SC2	FluA	FluB	SC2	FluA	FluB	SC2	FluA	FluB	
FluA 2X LoD	Mean Cq	N/A	36.91	N/A	N/A	36.70	N/A	N/A	35.93	N/A	N/A	35.83	N/A	40/40
	StdDev	N/A	0.90	N/A	N/A	0.39	N/A	N/A	0.37	N/A	N/A	0.51	N/A	
FluA 5X LoD	Mean Cq	N/A	35.14	N/A	N/A	35.33	N/A	N/A	34.50	N/A	N/A	34.69	N/A	40/40
	StdDev	N/A	0.17	N/A	N/A	0.43	N/A	N/A	0.25	N/A	N/A	0.15	N/A	
FluB 2X LoD	Mean Cq	N/A	N/A	36.78	N/A	N/A	37.08	N/A	N/A	37.02	N/A	N/A	36.20	40/40
	StdDev	N/A	N/A	0.42	N/A	N/A	0.69	N/A	N/A	0.68	N/A	N/A	0.52	
FluB 5X LoD	Mean Cq	N/A	N/A	35.52	N/A	N/A	35.71	N/A	N/A	35.08	N/A	N/A	35.36	40/40
	StdDev	N/A	N/A	0.73	N/A	N/A	0.43	N/A	N/A	0.72	N/A	N/A	0.38	
SC2 2X LoD	Mean Cq	36.13	N/A	N/A	37.06	N/A	N/A	36.31	N/A	N/A	36.57	N/A	N/A	40/40
	StdDev	0.43	N/A	N/A	0.45	N/A	N/A	0.59	N/A	N/A	0.60	N/A	N/A	
SC2 5X LoD	Mean Cq	34.72	N/A	N/A	34.35	N/A	N/A	35.14	N/A	N/A	35.05	N/A	N/A	40/40
	StdDev	0.39	N/A	N/A	0.25	N/A	N/A	0.29	N/A	N/A	0.27	N/A	N/A	
Negative	Mean Cq	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/40
	StdDev	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	

N/A; Negative, no detectable Cq value

Retrospective Clinical Evaluation

The performance of Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit with nasopharyngeal swab clinical samples was evaluated at an internal Bio-Rad site using clinical remnant individual samples collected from patients with signs and symptoms of an upper respiratory infection as follows:

- 32 influenza A and B negative samples as determined using a 510(k) cleared molecular assay (cleared within the last 5 years), which were confirmed to also be negative for SARS-CoV-2 with the Bio-Rad SARS-CoV-2 ddPCR Kit (EUA authorized molecular assay).
- 69 influenza A positive samples confirmed using a 510(k) cleared multi-analyte molecular assay
- 43 influenza B positive samples confirmed using a 510(k) cleared multi-analyte molecular assay
- 42 SARS-CoV-2 positive samples confirmed using EUA authorized molecular assays

The samples were collected by qualified personnel according to the package insert of the collection device and stored frozen at -80°C. The positive specimens represent a wide range of viral load and included low positive samples. No Cq/Ct values were available for these samples so these were all tested internally using an acceptable EUA RT-PCR assay to confirm that a representative range of clinical samples was tested and to serve as the adjudicator test in the event of a discrepancy between Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit result and the result from the Influenza A/B (IVD) or SARS-CoV-2 (EUA) test for a given sample.

Nucleic acid was purified from the 186 clinical samples using the QIAGEN QIAamp Viral RNA Mini kit using a sample volume of 140 µL and an elution volume of 60 µL. The samples were blinded, and assessed with Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit using the Bio-Rad CFX Opus 96 instrument. The Bio-Rad CFX Opus 96 is considered representative of all the instruments validated for use with the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit, as demonstrated during the LoD study.

Of the 186 unique clinical samples, there were 154 positive results (including both concordant and discordant positives) and 32 negative results. The 154 positive results were from 154 unique patient samples and the 208 concordant negatives were from 144 total sample results. The majority of the results of the clinical evaluation show concordant results with the comparator tests. All controls performed as expected. Data are presented in Table 45.

Table 45. Clinical Performance - Comparison of Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit with Other FDA Cleared/Authorized Comparator Methods

Analyte	Number of Samples	Test Results				Agreement Statistics		
		Concordant Positive (N)	Discordant Positive (N)	Concordant Negative (N)	Discordant Negative (N)	Agreement Parameter	Percent Agreement (%)	95% CI (LCL, UCL)*
Influenza A	144	69	0	75	0	PPA	100%	94.7%, 100%
						NPA	100%	95.1%, 100%
Influenza B	144	43	0	101	0	PPA	100%	91.8%, 100%
						NPA	100%	96.3%, 100%
SARS-CoV-2	74	41	1[a]	32	0	PPA	97.6%	87.7%, 99.6%
						NPA	100%	89.3%, 100%

PPA = Positive Percent Agreement, NPA = Negative Percent Agreement

CI = confidence interval; LCL = Lower confidence Limit; UCL = Upper confidence Limit

*Confidence interval is calculated using Wilson's Score method

[a] Discordant analysis was completed using an EUA-RT-PCR assay and was negative for SARS-CoV-2

References

1. Center for Disease Control and Prevention. Biosafety in Microbiological and Biomedical Laboratories, 5th Edition. 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. Center for Disease Control and Prevention. MMWR. Prevention and Control of Influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP), July 2008.
3. Clinical and Laboratory Standards Institute (CLSI). Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods. Approved Guideline-Second Edition. CLSI Document MM13-Ed2: Wayne, PA; CLSI, 2020.

4. Clinical and Laboratory Standards Institute (CLSI). Protection of Laboratory Workers from Occupationally Acquired Infections. Approved Guideline-Fourth Edition. CLSI Document M29-A4: Wayne, PA; CLSI, 2014.

Appendix A: Additional Label

For CFX96 Touch and CFX Opus 96 Real-Time PCR Systems

Please print and place this label on the front panel of the instrument. If the instruments include labeling indicating "For Research Use Only", please cover with the below "Emergency Use Only" labeling. The instrument should retain this labeling throughout the EUA use of the Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit.

Emergency Use Only

This instrument is authorized for use with Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit

Bio-Rad Reliance SARS-CoV-2/FluA/FluB RT-PCR Assay Kit

IVD, Rx-only, for use under EUA only
Download instruction for use at

bio-rad.com/SARS-CoV-2FluAFluB-IVD

Please contact Bio-Rad Laboratories (1-800-424-6723, option 2) if you require a printed copy free of charge

- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use in laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C.§263a, that meet requirements to perform high complexity tests;
- This product has been authorized only for the simultaneous qualitative detection and differentiation of nucleic acids from SARS-CoV-2, influenza A virus, and influenza B virus and not for any other viruses or pathogens; and
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3 (b)(1), unless the declaration is terminated, or authorization is revoked sooner.