Bio-Rad Reliance SARS-CoV-2 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 12014115

SARS-CoV-2 RT-PCR Oligos (1 Each)

Reliance One-Step Multiplex RT-qPCR Supermix (1 Each)

Exact Diagnostics SARS-CoV-2 Standard (2 Each)

Exact Diagnostics SARS-CoV-2 Negative (2 Each)

IVD

Bio-Rad Laboratories, Inc.
4000 Alfred Nobel Drive Hercules, CA USA 94547

10000132460 Rev. E

Translations

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

Symbols Lexicon

 European Conformity	 Manufacturer	 Authorized Representative in the European Union
 Lot Number	 Use by	 For In Vitro Diagnostic Use
 Temperature Limit	 Catalog Number	 Consult Instructions for Use
 Number of Tests	 For use with	 Serial Number
Rx Only Prescription Use Only	 Unique Device Identification – Device Identifier	 Contains Latex
 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.

Phone: 1-800-424-6723, option 2

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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 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 real-time reverse transcription polymerase chain reaction (RT-PCR) Assay Kit is intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal aspirates, and nasal washes from individuals suspected of COVID-19 by their healthcare provider. 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.

Results are for the identification of SARS-CoV-2 RNA, which is generally detectable in upper respiratory specimens during the acute phase of the infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or coinfection with other viruses. Laboratories within the United States and its territories are required to report all test results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

The Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit is intended for use by qualified clinical laboratory personnel specifically trained and instructed in RT-PCR techniques and in vitro diagnostic procedures.

The Bio-Rad Reliance SARS-CoV-2 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, assessment of the extent of the outbreak, monitoring of intervention strategies, and surveillance studies.

The Bio-Rad Reliance SARS-CoV-2 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 RNA from the SARS-CoV-2 virus in nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal aspirates, and nasal washes from patients who are suspected of COVID-19. 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 also be reported in accordance with local, state, and federal regulations. Performance is unknown in asymptomatic patients.

The oligonucleotide primers and probes for detection of SARS-CoV-2 are the same as those reported by the Center for Disease Control and Prevention (CDC) and were selected from regions of the virus nucleocapsid (N1 and N2) gene. The panel is designed for specific detection of the SARS-CoV-2 (two primer/probe sets). 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 RT-PCR Assay Kit can be used with the Bio-Rad CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, and CFX384 Touch Real-Time PCR Detection Systems, and the Thermo Fisher Scientific, Inc. Applied Biosystems (AB) 7500 Fast Real-Time PCR Instrument (Table 1). The workflow consists of four steps (Table 2).

Table 1. Instruments Required

Catalog Number	Product Name
1855485	CFX384 Touch Real-Time PCR Detection System[2]
1855195	CFX96 Touch Real-Time PCR Detection System[2]
12011319	CFX Opus 96 Real-Time PCR System[2]
12011452	CFX Opus 384 Real-Time PCR System[2]
4351106, 4351107	Applied Biosystems 7500 Fast Real-Time PCR System[1, 2]
1845097-IVD 1841000-IVD	CFX96 Dx ORM C1000 Dx Thermal Cycler

Starter packages available for CFX Touch and CFX Opus Systems

[1]This instrument requires qualification prior to use with the Bio-Rad Reliance SARS-CoV-2 EUA assay. Please refer to Appendix A for the required protocol and acceptance criteria

[2] Please refer to Appendix B of this IFU for EUO labeling that should be affixed to select instruments.

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

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

Workflow	
Step 1	Isolation of viral RNA from nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal aspirates, and nasal washes
Step 2	RT-PCR Plate Setup
Step 3	One-step reverse transcription and PCR
Step 4	Analysis

Reagents and Instruments

Materials Provided

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

Table 3. Materials Required Included in Kit for the Reliance SARS-CoV-2 RT-PCR Assay Kit

Product Name	QTY (Tubes)	Volume (µL)	Storage Conditions, °C
4x Reliance One-Step Multiplex Supermix	1	1000	-20°C
Exact Diagnostics SARS-CoV-2 Standard	2	300	-20°C
Exact Diagnostics SARS-CoV-2 Negative	2	300	-20°C
SARS-CoV-2 RT-PCR Oligos	1	300	-20°C

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

Materials Required but Not Provided

Reagents and Consumables:

Reagents for RNA Purification

The Thermo Fisher Scientific MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Catalog #A48310, #A42352) and the QIAGEN QIAamp Viral Mini Kit (Catalog #52906, #52904) are validated for use with the Reliance SARS-CoV-2 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 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, CFX96 Dx, CFX Opus 384, and CFX384 Touch 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
HSP3805, or equivalent*	Hard-Shell 384-Well PCR Plates, thin wall, skirted, clear/white	50	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 & 384-well colored shell/white well PCR plates*

Table 5. Materials Required but Not Provided for Running on the Applied Biosystems 7500 Fast 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 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 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 or 384-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 professional use only.
3. This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories;
4. This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens; and,
5. 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.
6. Positive results are indicative of the presence of SARS-CoV-2 RNA.
7. Laboratories within the United States and its territories should report all test results to the appropriate public health authorities, as required.
8. 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]
9. 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.
10. 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.
11. Optimize workflow and space to minimize the risk of carryover contamination from completed PCR reactions.
12. Ensure that the real-time PCR system and automation system have a dedicated space in separate areas to avoid amplicon contamination.

13. Perform assay setup and template addition in different locations, with dedicated pipettors.
14. Use proper laboratory safety procedures for working with chemicals and handling specimens.
15. Change gloves frequently when transporting and working with different reagents.
16. Failure to follow the procedures and conditions described in this document can cause incorrect results and adverse effects.
17. Do not substitute Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit reagents with other reagents.
18. Setup and template addition must be performed under RNase/DNase-free conditions.
19. It is required that the AB7500 Fast instrument is appropriately qualified prior to use with the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit using established testing protocols. Refer to the protocol provided in Appendix A.
20. Ensure that regular maintenance and calibration are performed on all equipment according to 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 diethyl pyrocarbonate (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.

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-A 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 a delay in extraction is expected, store specimens at -70°C or lower
    - Extracted nucleic acid should be stored at 4°C if it is to be used within 4 hours, or -70°C or lower if stored longer than 4 hours

Use of Control Materials

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

- A no-template control (NTC) is needed to detect reagent and/or environmental contamination. An NTC uses RNase/DNase-free water in place of a clinical specimen sample with a minimum of one well per reaction plate.
- A positive control is needed to detect substantial reverse transcriptase and/or reagent failure, including primer and probe integrity. The test utilizes Exact Diagnostics SARS-CoV-2 Standard, which is manufactured with synthetic RNA transcripts containing five gene targets: *E, N, ORF1ab*, *RdRP, and S* Genes of SARS-CoV-2, each quantified at 200,000 copies/mL along with human genomic DNA background. This control material is spiked into a sample-like matrix to achieve a final concentration of 1,000 copies/mL and the nucleic acid is extracted. One positive control must be included per batch of samples extracted, with a minimum of one positive control well per reaction plate.
- A negative control is needed to detect extraction step failure or reagent/environmental contamination. The test utilizes Exact Diagnostics SARS-CoV-2 Negative control, which is manufactured with human genomic DNA and RNA. This control material is spiked into a sample-like matrix and the nucleic acid is extracted. One negative control must be included per batch of samples extracted, with a minimum of one negative control well per reaction plate.

Reagent Handling and Storage

Reliance SARS-CoV-2 RT-PCR Assay Kit

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

Work Areas

All necessary safety precautions should be taken according to good 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 from laboratory equipment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything on cold blocks when possible 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. Make sure 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 RT-PCR Assay Kit Protocol

Overview

The Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit is intended for the qualitative detection of RNA from the SARS-CoV-2 in upper respiratory tract specimens, including nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, anterior nasal swabs, mid-turbinate nasal swabs, nasal aspirates, and nasal washes. The assay detects two regions of the SARS-CoV-2 nucleocapsid gene (*termed N1 and N2*) and a constitutively expressed human *RP* gene, all in one reaction. Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

The test is composed of two principal steps: (1) extraction of RNA from patient specimens and (2) one-step reverse transcription and polymerase chain reaction amplification and detection of the SARS-CoV-2 specific *N1 and N2* targets, which detect viral infection, and the RP assay that detects background human nucleic acid in the patient specimen.

Description of Test Steps

Nucleic acids are isolated and purified from upper respiratory tract specimens, using the Thermo Fisher Scientific MagMAX Viral/Pathogen Nucleic Acid Isolation Kit or the QIAGEN QIAamp Viral RNA Mini Kit, following the manufacturer's instructions. The purified nucleic acids are reverse transcribed and amplified using Reliance One-Step Multiplex RT-qPCR Supermix. The SARS-CoV-2 RT-PCR Oligos contains a blend of the primers and probes for SARS-CoV-2 targets (*N1* and *N2)* and the human *RP* gene to enable multiplexed detection of the targets.

Nucleic Acid Extraction

Performance of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit is dependent upon the amount and quality of template RNA purified from human specimens. The following commercial extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the test:

- Thermo Fisher Scientific MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Catalog #A48310, #A42352)
- QIAGEN QIAamp Viral RNA Mini Kit (Catalog #52906, #52904)

Follow the manufacturer's recommended procedures for sample extraction. A positive control and a negative control must be included in each extraction batch.

Preparation of controls

Positive control: Spike 5 µL of Exact Diagnostics SARS-CoV-2 Standard into a tube containing 995 µL of phosphate buffered saline (PBS). Treat as a patient sample and process for nucleic acid extraction together with other samples according to the manufacturer's instructions.

Negative control: Spike 5 µL of Exact Diagnostics SARS-CoV-2 Negative control into a tube containing 995 µL of PBS. Treat as a patient sample and process for nucleic acid extraction together with other 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 Supermix is viscous. It is critical to vortex before beginning the assay mix preparation.*

4. RT-PCR master mix preparation:
   a. Prepare a master mix according to the number of patient samples and controls to be tested plus 10% more volume (Table 7) when more than 1 sample is 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 RT-PCR 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 a Microseal 'B' PCR Plate 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 CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, CFX384 Touch, or AB7500 Fast Real-Time PCR instrument.

Bio-Rad CFX Instrument Setup

The following instructions are for running the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit on a computer-controlled CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, or CFX384 Touch Real-Time PCR Systems. For more detailed information, refer to the instrument manuals and addendum.

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

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

Cycling Protocol Setup for CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, and CFX384 Touch

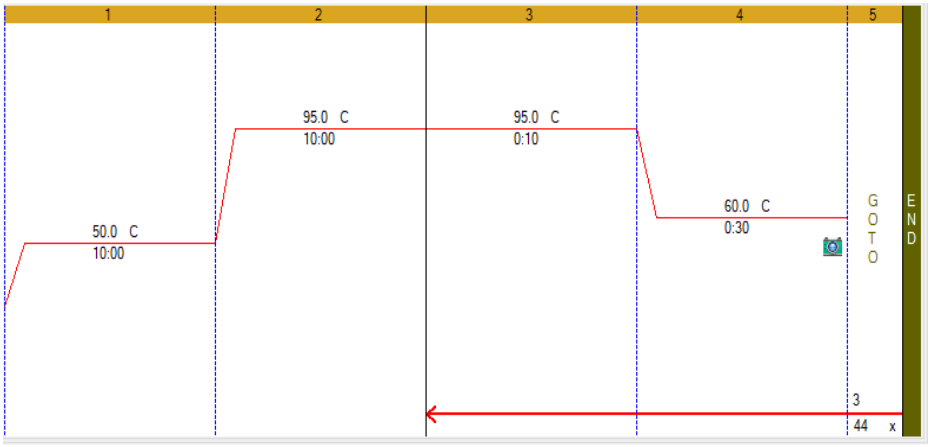
1. Click **File** -> **New** -> **Protocol** in the menu bar to open the Protocol Editor
2. Change Sample Volume to 20 µl
3. Modify the Cycling Protocol to the guidelines in Table 8 below:

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: Final Cycling Protocol



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

Plate Setup for CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, and CFX384 Touch

1. Click **File** -> **New** -> **New Plate** in the menu bar to open the Plate Editor
2. Select **Settings** -> **Plate Size** -> select 96 wells or 384 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, and Texas Red 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 (N1)
   HEX – SARS-CoV-2 (N2)
   Texas – RNase P
9. Save the file by clicking **File -> Save As**
10. Name the plate file "**Bio-Rad SARS-CoV-2 RT-qPCR Plate Setup**"
11. Close the file by clicking **File -> Close**

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

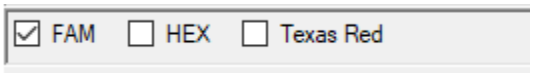
1. Select the instrument from the Select Instrument drop-down menu in the Startup Wizard
2. Click User-defined in the Select run type section of the Startup Wizard. This will open the Run Setup panel.
3. Click **Select Existing** in the protocol tab
4. Select the cycling protocol file "**Bio-Rad SARS-CoV-2 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 RT-qPCR 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

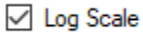
The run data file will open automatically after the run has completed. To open a file that has been closed, click **File** -> **Open** -> **Data File** -> Select the data file from the menu.

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

1. Click Settings -> Cycles to Analyze -> enter "5" in the first cell to replace the default setting of "1"
2. Deselect HEX and Texas Red by unchecking the corresponding boxes under the amplification plot. Only the FAM box should be selected.



3. Select Log Scale by checking the box in the lower right of the amplification plot



4. Visually inspect the traces. Any well with amplification in the FAM channel should show an exponential increase in RFU values up until the reaction plateaus.
5. Manual baseline adjustment may be necessary if amplification traces are not exponential. To manually define the baseline, select **Settings -> Baseline Threshold**. Highlight the well to be adjusted, enter 2 in the **Baseline Begin** cell, enter a cycle number that is 2 cycles before amplification trace starts to rise in the **Baseline End** cell. Click **OK** to apply.
6. Set the FAM threshold in the amplification plot by clicking and dragging the threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal.
7. Confirm baseline and define the threshold for the HEX and Texas Red channels by selecting the appropriate fluorophore in Step 2 and repeating the process defined above

Applied Biosystems 7500 Fast Real-Time Instrument Setup

The following instructions are essential for running Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit on a AB7500 Fast Real-Time PCR system. For more detailed information about plate, and cycling protocol setup, refer to the Applied Biosystems 7500 Fast Real-Time PCR instrument manual.

1. Launch the 7500 software
2. Select **File** -> **New** in 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	*N1*
HEX	*N2*
TEXAS RED	*RP*

Note: For the *N2* assay, the VIC channel is applicable for detecting this target. Alternatively, the HEX reporter can be added by calibrating the instrument. This can be done by following the instrument manual.

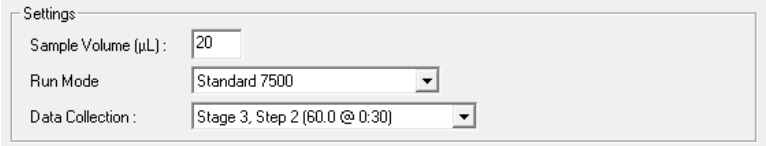
5. Select **Passive Reference** -> **None**
6. Define the cycling protocol using the values listed in Table 10

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 3.

Figure 3: Data Collection Dropdown Menu for AB7500



Data Analysis on Applied Biosystems 7500 Fast Real-Time

The following instructions are essential for analyzing results obtained using Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit with the 7500 Fast 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 the **Result** tab at the upper left corner of the software.
3. Click on the **Amplification Plot** tab
4. Highlight all the samples from the run to view all amplification curves.
5. Set **Data** to **Delta Rn vs. Cycle** on the right side of the panel
6. Set **Detector** to **N1**
7. Set **Line Color** to **Detector Color**
8. Select **Manual Ct** and **Manual Baseline** under **Analysis Settings**. Do not change the Manual Baseline default numbers.
9. Click and drag the threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal.
10. Click the **Analyze** button in the lower right corner of the window. The red threshold will turn to green, indicating the data has been analyzed.
11. Repeat step 6-10 to analyze results for each set of markers

Interpretation of Results

The NTC, positive control, and negative control should be examined prior to interpreting the patient results. If the controls are not valid, the patient results cannot be interpreted.

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

No Template Control (NTC)

The NTC reactions for the SARS-CoV-2 RT-PCR oligo mix should not exhibit positive signals in any channel (FAM, HEX or Texas Red) for any of the three targets tested, *N1, N2, or RP*. If any of the NTC reactions exhibit positivity, 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 re-test all samples that were included in that batch.

Positive Control

The Positive Control will yield positive results (Cq < 40) for the detection of *N1, N2 and, RP* primer and probe sets.

Negative Control

The Negative Control should yield a positive result with the *RP* primer and probe set (Cq < 40) and negative results with all SARS-CoV-2 *N1 and N2* targets.

Examination and Interpretation of Patient Specimen Results

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. The expected performance of the Bio-Rad Reliance SARS-CoV-2 RT-PCR assay controls is shown in Table 11.

Table 11. Expected Performance of Controls in the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit

Control Type	External Control Name	Used to Monitor	SARS-CoV-2		Internal Control	Expected Cq		
			N1	N2	RP	N1	N2	RP
NTC	RNase/DNase free water	Reagent and/or environmental contamination	Negative	Negative	Negative	Cq ≥ 40 or N/A		
Negative	SARS-CoV-2 Negative	Reagent and/or Environment Contamination	Negative	Negative	Positive	Cq ≥ 40 or N/A	Cq ≥ 40 or N/A	< 40
Positive	SARS-CoV-2 Standard	Substantial reagent failure, including primer and probe integrity	Positive	Positive	Positive	< 40	< 40	< 40

If any control does not meet these criteria, the test may have improper setup or execution, or reagent or equipment malfunction or failure. Invalidate the run and re-test.

RP (Internal Control)

All clinical samples should exhibit positive signals with the *RP* primers and probe (Cq < 40), thus indicating the presence of the human *RP* gene. A 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 the SARS-CoV-2 *N1* and/or *N2* is/are positive, even in the absence of a positive *RP*, the result should be considered valid. It is possible that 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 SARS-CoV-2 virus RNA in a clinical specimen.
- If all SARS-CoV-2 markers and *RP* are negative for the specimen, the result should be considered invalid for the specimen. If a residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-testing, report the results as invalid and a new specimen should be collected.

SARS-CoV-2 Markers (*N1 and N2*)

- SARS-CoV-2 is detected when all controls exhibit the expected performance; a specimen is considered positive for SARS-CoV-2 if all SARS-CoV-2 marker (*N1, N2*) amplification curves cross the threshold line within 40 cycles. The *RP* may or may not be positive as described above, but the SARS-CoV-2 result is still valid.
- SARS-CoV-2 is not detected when all controls exhibit the expected performance and all SARS-CoV-2 markers (*N1, N2*) amplification curves DO NOT cross the threshold line within 40 cycles AND the RNase P amplification curve DOES cross the threshold line within 40 cycles.
- The result is inconclusive when all controls exhibit the expected performance and the amplification curves for either of the SARS-CoV-2 markers (N1 or N2, but not both markers) cross the cycle threshold within 40 cycles. The extracted RNA should be retested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained, report the inconclusive result. Consult with your state public health laboratory or CDC, as appropriate, to request guidance and/or to coordinate transfer for the specimen for additional analysis.
- The result is invalid when all controls exhibit the expected performance and the amplification curves for the SARS-CoV-2 markers (*N1, N2*) AND the *RP* marker DOES NOT cross the cycle threshold within 40 cycles. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from the residual specimen and re-test. If the re-tested sample is negative for all markers and *RP*, the result is invalid and collection of a new specimen from the patient should be considered.

For ease of interpretation, refer to the guide in Table 12.

Table 12. Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit Results Interpretation Guide

SARS-CoV-2 N1 Result	SARS-CoV-2 N2 Result	Internal Control RP Result	Interpretation	Actions
Positive (Cq < 40)	**Positive** (Cq < 40)	**Positive or Negative**	SARS-CoV-2 detected	Store samples at -70°C as needed and report the results to the appropriate Public Health Authority
If only one of the two targets is positive (Cq < 40)		**Positive or Negative**	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat RT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
Negative (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Positive** (Cq < 40)	SARS-CoV-2 not detected	Report the results to the appropriate Public Health Authority
Negative (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	**Negative** (Cq ≥ 40 or N/A)	Invalid Result	Repeat extraction and RT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

Limitations

1. The Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit has only been evaluated for use on the CFX Opus 96, CFX96 Touch, CFX96 Dx, CFX Opus 384, CFX384 Touch, and Applied Biosystems 7500 Fast Real-Time PCR Systems.
2. Performance of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit has only been established in nasopharyngeal swab specimens. Use of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit with other specimen types has not been assessed and performance characteristics are unknown.
3. Oropharyngeal swabs, nasal swabs, mid-turbinate swabs, nasal aspirates and nasal washes are considered acceptable specimen types for use with the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit but performance with these specimen types has not been established.
4. Reliable results depend on proper sample collection, storage, and handling procedures.
5. This test is used for the detection of SARS-CoV-2 RNA in upper respiratory specimens collected in a Universal Transport Medium (UTM) or Universal Viral Transport System (UVT). Testing of other sample types with Reliance SARS-CoV-2 RT-PCR Assay Kit may result in inaccurate results.
6. Detection of SARS-CoV-2 RNA may be affected by sample collection methods, patient factors (for example, presence of symptoms), and/or stage of infection.
7. The output of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit is a qualitative assessment of patient samples that are positive of SARS-CoV-2. The user assesses RT-PCR results for controls and patient samples to make a qualitative call of SARS-CoV-2 detected or not detected. The values reported should not be used or interpreted as quantitative.
8. As with any molecular test, mutations within the target regions of Reliance SARS-CoV-2 RT-PCR Assay Kit could affect primer and/or probe binding resulting in a failure to detect the presence of a virus.
9. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next, users perform method correlation studies in their laboratory to qualify technology differences. One hundred percent agreement between the results should not be expected due to the aforementioned differences between technologies. Users should follow their own specific policies/procedures.

10. The performance of this test was established based on the evaluation of a limited number of clinical specimens. 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.

Conditions of Authorization for the Laboratory

The Bio-Rad Reliance SARS-CoV-2 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/in-vitro-diagnostics-euas

However, to assist clinical laboratories using the Bio-Rad Reliance SARS-CoV-2 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 will 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

Limit of detection (LOD) studies were conducted to determine the lowest detectable concentration of SARS-CoV-2 at which greater or equal to 95% of all (true positive) replicates test positive using the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit. To determine the LOD, patient samples were simulated for the study through titrating a synthetic virus (AccuPlex SARS-COV-2, Seracare, Cat# 0505-0126) into a background of pooled SARS-CoV-2 negative nasopharyngeal swab matrix prior to nucleic acid purification. The Thermo Fisher MagMAX Viral/Pathogen Nucleic Acid Isolation Kit and QIAGEN QIAamp Viral RNA Mini Kit were the extraction kits validated in the LOD study.

For LOD studies using the QIAGEN QIAamp Viral RNA Mini Kit, simulated patient samples were produced with viral loads of 31.25, 62.5, 125 and 250 copies per mL for the CFX96 Touch, CFX96 Dx, CFX384 Touch and AB7500 instruments. In a subsequent study to include the CFX Opus 96 and CFX Opus 384, the viral loads tested for LOD studies was adjusted to 125, 250, 500 and 1000 copies per mL. Twenty replicates of each viral dilution were isolated according to the manufacturer's instructions (140 µL sample volume and an elution volume of 60 µL). The LOD results are shown in Table 13. CFX96 Touch and CFX96 Dx instruments can detect SARS-CoV-2 infection with an LOD of 125 viral copies/mL. CFX384 Touch, CFX Opus 96, CFX Opus 384, and AB7500 instruments can detect SARS-CoV-2 infection with an LOD of 250 viral copies/mL.

Subsequent to the QIAGEN QIAamp Viral RNA Mini Kit LOD study, the LOD studies on all instruments using Thermo Fisher’s MagMAX Viral/Pathogen Nucleic Acid Isolation Kit used simulated patient samples that were produced with viral loads of 125, 250, 500 and 1000 copies per mL and isolated 20 replicates of each viral dilution according to the manufacturer's instructions (200 µL sample volume and an elution volume of 100 µL). The LOD results are shown in Table 14. CFX96 Dx and CFX Opus 96 instruments can detect SARS-CoV-2 infection with an LOD of 125 viral copies/mL. CFX96 Touch, CFX Opus 384, and AB7500 instruments can detect SARS-CoV-2 infection with an LOD of 250 viral copies/mL. The CFX384 Touch instrument can detect SARS-CoV-2 infection with an LOD of 500 viral copies/mL.

The LOD range is 125-250 viral copies/mL on all instruments Independent of nucleic acid purification method (Table 15) with the exception of CFX384 Touch, which has an LOD of 500 copies/mL when used with the Thermo Fisher’s MagMAX Viral/Pathogen Nucleic Acid Isolation Kit.

Table 13. LOD Results QIAamp Viral RNA Mini Kit Extracted Samples

SARS-CoV-2 copies/mL	CFX96 Touch		CFX96 Dx		CFX384 Touch		AB7500	
	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates
250	20/20	20/20	20/20	20/20	20/20	20/20	20/20	20/20
125	19/20	19/20	19/20	20/20	18/20	19/20	16/20	15/20
62.5	20/20	17/20	19/20	18/20	18/20	18/20	16/20	12/20
31.25	17/20	19/20	14/20	15/20	13/20	10/20	9/20	7/20

Table 13 (cont.) LOD Results QIAamp Viral RNA Mini Kit Extracted Samples

SARS-CoV-2 copies/mL	CFX Opus 96		CFX Opus 384	
	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 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	19/20	19/20
125	16/20	19/20	17/20	13/20

Table 14. LOD Results MagMAX Viral/Pathogen Nucleic Acid Isolation Kit Extracted Samples

SARS-CoV-2 copies/mL	CFX96 Touch		CFX96 Dx		CFX384 Touch		AB7500	
	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates
1000	20/20	20/20	20/20	20/20	19/20	20/20	20/20	20/20
500	20/20	20/20	20/20	20/20	19/20	20/20	19/20	20/20
250	20/20	20/20	19/20	20/20	12/20	20/20	20/20	19/20
125	15/20	19/20	19/20	20/20	10/20	10/20	14/20	14/20

Table 14 (cont.) LOD Results MagMAX Viral/Pathogen Nucleic Acid Isolation Kit Extracted Samples

SARS-CoV-2 copies/mL	CFX Opus 96		CFX Opus 384	
	N1 Positive replicates /Total replicates	N2 Positive replicates /Total replicates	N1 Positive replicates /Total replicates	N2 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	19/20	19/20
125	20/20	19/20	20/20	9/20

Table 15. Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit LOD Summary

Instrument	QiaAMP Viral Mini Kit	MagMAX Viral/Pathogen Kit
CFX96 Touch	125 copies/mL	250 copies/mL
CFX96 Dx	125 copies/mL	125 copies/mL
AB7500	250 copies/mL	250 copies/mL
CFX384 Touch	250 copies/mL	500 copies/mL
CFX Opus 96	250 copies/mL	125 copies/mL
CFX Opus 384	250 copies/mL	250 copies/mL

Inclusivity

The Bio-Rad Reliance SARS-CoV-2 RT-PCR Oligos (primers and probes) sequences for *N1, N2, and RP* were developed by the CDC. CDC performed an alignment with the oligonucleotide primer and probe sequences of the CDC 2019 nCoV Real Time RT-PCR Diagnostic Panel 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) database as of June 20, 2020 to demonstrate the predicted inclusivity of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic panel. An evaluation of 31,623 available SARS-CoV-2 sequences in GISAID was used in this study. With the exception of one nucleotide mismatch with frequency > 1% (2.00%) at the third position of the N1 probe, the frequency of all mismatches was < 1%, indicating that prevalence of the mismatches were sporadic. Only one sequence (0.0032%) had two nucleotide mismatches in the N1 probe, and one other sequence from a different isolate (0.0032%) had two nucleotide mismatches in the N1 reverse primer. No sequences were found to have more than one mismatch in any N2 primer/probe region.

The risk of a single mismatch resulting in a significant loss in reactivity, and false-negative result, is low due to the design of the primers and probes with melting temperatures > 60°C and run conditions of the assay with annealing temperature at 55°C to tolerate one to two mismatches.

Analytical Specificity (cross-reactivity)

In silico analysis for the pathogens listed in Table 16 was performed by downloading one GenBank reference sequence per genome for each of the organisms. The reference sequences were compared against the Bio-Rad SARS-CoV-2 targets, N1 and N2 for all possible combinations (forward primer, reverse primer, probe, and the reverse complements for all of these) to determine homology percentage. If any of these primer combinations were mapped to a sequence on opposite strands with a homology of >80% on the same target within a short distance (≤100 bp) apart, potential amplifications were flagged. No

potential unintended cross-reactivity is expected based on this in silico analysis except for SARS-coronavirus (SARS-CoV) with the *N2* target.
As reported under the CDC EUA, the in-silico analysis for the *N1* primer/probe set showed high sequence homology of the *N1* probe with SARS-CoV and bat SARS-like coronavirus genome. However, forward and reverse primers showed no sequence homology with SARS-CoV and bat SARS-like coronavirus genome. Combining the primers and probe results, there are no significant homologies with the human genome, other coronaviruses, or human microflora that would predict potential false positive RT-PCR results.

Table 16. In silico analysis for SARS-CoV-2

Pathogens Tested in-Silico	Unintended Cross-Reactivity to N1	Unintended Cross-Reactivity to N2
SARS-CoV	None detected	Homology match 92%*
MERS-coronavirus	None detected	None detected
Human adenovirus A	None detected	None detected
Human adenovirus B1	None detected	None detected
Human adenovirus B2	None detected	None detected
Human adenovirus C	None detected	None detected
Human adenovirus D	None detected	None detected
Human adenovirus E	None detected	None detected
Human adenovirus F	None detected	None detected
Human Metapneumovirus (hMPV)	None detected	None detected
Parainfluenza virus 1	None detected	None detected
Parainfluenza virus 2	None detected	None detected
Parainfluenza virus 3	None detected	None detected
Parainfluenza virus 4	None detected	None detected
Influenza A H3N2	None detected	None detected
Influenza A H2N2	None detected	None detected
Influenza A H7N9	None detected	None detected
Influenza A H1N1	None detected	None detected
Influenza B	None detected	None detected
Human enterovirus A	None detected	None detected
Human enterovirus B	None detected	None detected
Enterovirus E, Bovine enterovirus	None detected	None detected
Enterovirus F	None detected	None detected
Enterovirus G, Porcine enterovirus 9	None detected	None detected
Enterovirus H, Simian enterovirus A	None detected	None detected
Enterovirus J strain 1631	None detected	None detected
Enterovirus J strain N203	None detected	None detected
Respiratory syncytial virus	None detected	None detected
Rhinovirus A, Human rhinovirus 89	None detected	None detected
Rhinovirus A, Human rhinovirus 1 strain ATCC VR-1559	None detected	None detected

Pathogens Tested in-Silico	Unintended Cross-Reactivity to N1	Unintended Cross-Reactivity to N2
Rhinovirus B	None detected	None detected
Rhinovirus C, Human rhinovirus C	None detected	None detected
Rhinovirus C, Human rhinovirus NAT001	None detected	None detected
Haemophilus influenzae	None detected	None detected
Legionella pneumophila	None detected	None detected
Mycobacterium tuberculosis	None detected	None detected
Streptococcus pneumoniae	None detected	None detected
Streptococcus pyogenes	None detected	None detected
Enterovirus (e.g. EV68)	None detected	None detected
Pneumocystis jirovecii	None detected	None detected

**Analysis of the forward primer of the N2 target showed high homology to bat SARS-like coronaviruses. However, the reverse primer and probe sequences showed no significant homology with the human genome, other coronaviruses or human microflora that would predict potential false-positive RT-PCR results. Combining the primers and probe results, there is no prediction of potential false-positive RT-PCR results.*

In addition to the *in silico* analysis, CDC reported analytical specificity and exclusivity demonstrating expected results are obtained for each organism noted in Table 17 to demonstrate that final results are not impacted by these viruses.

Table 17. Specificity/Exclusivity reported by CDC

Virus	Strain	Source	2019-nCoV_ N1	2019-nCoV_ N2	Final Result
Human coronavirus	229E	Isolate	0/3	0/3	Neg.
Human coronavirus	OC43	Isolate	0/3	0/3	Neg.
Human coronavirus	NL63	Clinical Specimen	0/3	0/3	Neg.
Human coronavirus	HKU1	Clinical Specimen	0/3	0/3	Neg.
MERS-coronavirus		Isolate	0/3	0/3	Neg.
SARS-coronavirus		Isolate	0/3	0/3	Neg.
Bocavirus		Clinical Specimen	0/3	0/3	Neg.
Mycoplasma pneumoniae		Isolate	0/3	0/3	Neg.
Streptococcus		Isolate	0/3	0/3	Neg.
Influenza A(H1N1)		Isolate	0/3	0/3	Neg.
Influenza A(H3N2)		Isolate	0/3	0/3	Neg.
Influenza B		Isolate	0/3	0/3	Neg.
Human adenovirus, type 1	Ad71	Isolate	0/3	0/3	Neg.
Human metapneumovirus		Isolate	0/3	0/3	Neg.
Respiratory syncytial virus	Long A	Isolate	0/3	0/3	Neg.
Rhinovirus		Isolate	0/3	0/3	Neg.
Parainfluenza 1	C35	Isolate	0/3	0/3	Neg.
Parainfluenza 2	Greer	Isolate	0/3	0/3	Neg.
Parainfluenza 3	C-43	Isolate	0/3	0/3	Neg.
Parainfluenza 4	M-25	Isolate	0/3	0/3	Neg.

Clinical Evaluation

The performance of Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit with nasopharyngeal swab clinical samples was evaluated using 34 individual negative clinical samples and 34 confirmed positive clinical samples. Samples procured by iSpecimen (Lexington, MA) were collected from patients with signs and symptoms of an upper respiratory infection. 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 represented a wide range of viral load and included low positive samples. The samples were provided with results obtained using a high sensitivity, EUA authorized molecular comparator assay.

Nucleic acid was purified from the 68 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 randomized, blinded, and assessed with Bio-Rad's Reliance SARS-CoV-2 RT-PCR Assay Kit using Bio-Rad's CFX384 Touch instrument, which had the highest LOD of the instruments tested. The QIAGEN QIAamp Viral RNA Mini extraction method was utilized for the clinical evaluation study because the LOD was within 2X of the LoD established with the MagMAX Viral/Pathogen Nucleic Acid

Isolation Kit extraction method across tested instruments. Results obtained from samples tested using the Bio-Rad's Reliance SARS-CoV-2 RT-PCR Assay Kit were compared to the results obtained from the EUA authorized molecular comparator assay.

Clinical study results (Table 18) show a 100% percent positive agreement (PPA) with a 95% confidence interval of 89.9% - 100% and a 100% negative percent agreement (NPA) with a 95% confidence interval of 89.9% - 100%.

Table 18. PPA and NPA of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit against comparator

Reliance SARS-CoV-2 RT-PCR	Comparator Test Positive	Comparator Test Negative	Total	PPA [95% CI]	NPA [95% CI]
Test positive	34	0	34	100% [89.9%-100%]	100% [89.9%-100%]
Inconclusive	0	0	0		
Test negative	0	34	34		
Total	34	34	68		

There were two discordant results (Case ID 751 & 761) on initial testing; the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit was inconclusive and the molecular comparator assay was negative. Both samples were re-extracted and re-tested in three replicates using the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit as per the assay instructions for use. Repeat testing of the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit resolved the inconclusive result and confirmed the sample was negative. Additionally, both of the samples (Case ID 751 & 761) were confirmed negative by two additional EUA authorized molecular comparator assays.

FDA SARS-CoV-2 Reference Panel Testing

The evaluation of sensitivity and MERS-CoV cross-reactivity was performed using reference material (T1), blinded samples and a standard protocol provided by the FDA. The study included a range finding study and a confirmatory study for LoD. Blinded sample testing was used to establish specificity and to corroborate the LoD. The extraction method and instrument used were QIAGEN QIAamp Viral RNA Mini and the CFX384 Touch instrument. The results are summarized in Table 17.

Table 17: Summary of LoD Confirmation Result Using the FDA SARS-CoV-2 Reference Panel

Reference Materials Provided by FDA	Specimen Type	Product LoD	Cross-Reactivity
SARS-CoV-2	Nasopharyngeal Swab	$1.8x10^3$ NDU/mL	N/A
MERS-CoV		N/A	ND

NDU/mL: RNA NAAT detectable units/mL
N/A: Not Applicable
ND: Not Detected

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. 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: AB7500 Real-Time PCR System Qualification

Purpose

This appendix is intended to provide a qualification procedure describing how to prepare a panel of mock specimens for use in verifying performance of the AB7500 Fast Real-Time PCR System with the Bio-Rad Reliance SARS-CoV-2 RT-PCR Assay Kit by the end user. Qualification of the AB7500 Fast Real-Time PCR System with the Bio-Rad Reliance SARS-CoV-2 Assay Kit must be achieved prior to usage for diagnostic testing.

Required Materials

Description	Quantity	Included in the kit
Exact Diagnostics SARS-CoV-2 Standard	1 vial	Yes
Exact Diagnostics SARS-CoV-2 Negative	1 vial	Yes
Phosphate buffered saline (PBS, pH 7.4)	3 mL*	No

*The quantity indicated is for preparing one set of 9 mock specimens

Precautions

The positive control provided with the Reliance SARS-CoV-2 RT-PCR Assay Kit is comprised of RNA transcript encoding the SARS-CoV-2 (SC2) N1 and N2 genes and human genomic DNA for the RNaseP (RP) gene amplification. This control is non-infectious. Both the Exact Diagnostics SARS-CoV-2 Standard and Negative controls are single use only; do not refreeze. The Reliance SARS-CoV-2 RT-PCR Assay Kit should be handled in accordance with Good Laboratory Practices.

The control materials and other kit components must be stored at appropriate temperatures, as described in the Instruction for Use (IFU) and be kept on ice once thawed. The extracted RNA samples should be kept cold during preparation and use.

Instructions for Preparing Mock Specimens Before Extraction with the QIAGEN QIAamp Viral RNA Mini Kit

1. Prepare sufficient Buffer AVL (with carrier RNA) for 9 samples, according to manufacturer's instructions.
2. Label three 1.5ml RNase-free tubes as A, B, and C. Label nine 1.5 ml tubes as 1-9.
3. Aliquot 995 µL of PBS into Tube "A" then add 5 µL of Exact Diagnostics SARS-CoV-2 Standard. Mix well.
4. Aliquot 900 µL of PBS into Tube "B" then add 100 µL of Tube "A". Mix well.
5. Aliquot 995 µL of PBS into Tube "C" then add 5 uL of Exact Diagnostics SARS-CoV-2 Negative. Mix well.
6. Aliquot 560 µL of Buffer AVL containing carrier RNA into each of nine tubes labeled 1-9.
7. Add 140 µL of Tube A into each of the tubes 1-3.
8. Add 140 µL of Tube B into each of the tubes 4-6.
9. Add 140 µL of Tube C into each of the tubes 7-9.

10. Extract the samples using the QIAamp Viral RNA Mini Kit following the manufacturer's instructions, eluting the samples in 60 uL of AVE.

Instructions for Preparing Mock Specimens Before Extraction with the Thermo Fisher MagMAX Viral/Pathogen Nucleic Acid Isolation Kit

1. Label three 1.5ml RNase-free tubes as A, B, and C.
2. Aliquot 995 µL of PBS into Tube "A" then add 5 µL of Exact Diagnostics SARS-CoV-2 Standard. Mix well.
3. Aliquot 900 µL of PBS into Tube "B" then add 100 µL of Tube "A". Mix well.
4. Aliquot 995 µL of PBS into Tube "C" then add 5 uL of Exact Diagnostics SARS-CoV-2 Negative
5. Aliquot 10 µL of Proteinase K from the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit into each of nine wells, designated 1-9, of a deep-well 96-well plate
6. Add 200 µL of tube A into each of the wells 1-3.
7. Add 200 µL of tube B into each of the wells 4-6.
8. Add 200 µL of tube C into each of the wells 7-9.
9. Extract the samples using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit following the manufacturer's instructions, eluting the samples in 100 µL of Elution Solution.

Test of extracted samples

Follow the Reliance SARS-CoV-2 RT-PCR Kit IFU for testing each of the moderate concentration, low concentration, and negative samples at least once using the AB7500 Fast Real-Time PCR System.

Expected Results

Tubes 1-3 and wells 1-3 contain moderate concentration of SC2 (approximately at 4xLoD) and should be positive for N1, N2, and RP.

Tubes 4-6 and wells 4-6 contain low concentration of SC2 (approximately at 0.4xLoD) and should be positive for N 1, N2, and RP.

Tubes 7-9 and wells 7-9 are negative samples and should be negative for N1 and N2 but positive for RP.

Acceptance Criteria:

Negative samples (Tubes 7-9): 100% (3/3) should be in agreement with expected results.

Moderate Positive Samples (Tubes 1-3): 100% (3/3) should be in agreement with expected results

Low Positive Samples (Tubes 4-6): At least 66% (2/3) should be in agreement with expected results.

Successful qualification is required prior to use of the AB7500 Fast Real-Time PCR System with the Reliance SARS-CoV-2 RT-PCR Assay Kit for diagnostic testing.

Questions

If you have questions or comments about these instructions, please contact Bio-Rad Technical Support, which is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific Time. (US).

Phone: 1-800-424-6723, option 2

Email: Support@Bio-Rad.com (U.S./Canada only)

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.

Appendix B: Additional Label

For CFX96 Touch, CFX384 Touch, CFX Opus 96, CFX Opus 384, and Qualified* AB7500 Fast 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 RT-PCR Assay Kit.

* Refer to Appendix A: AB7500 Real-Time PCR System Qualification for instructions

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Emergency Use Only

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