



Smart Detect™ SARS-CoV-2 rRT-PCR Kit

**Instructions for Use
For Emergency Use Authorization (EUA)
Only
For *In Vitro* Diagnostic (IVD) Use
Rx Only**

INTENDED USE

Smart Detect™ SARS-CoV-2 rRT-PCR Kit is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) in human nasopharyngeal swab, anterior nasal swab and mid-turbinate nasal swab specimens 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, to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of 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 co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive 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 Smart Detect™ SARS-CoV-2 rRT-PCR Kit is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time RT-PCR and *in vitro* diagnostic procedures. The Smart Detect™ SARS-CoV rRT-PCR Kit is only for use under the Food and Drug Administration's Emergency Use Authorization (EUA).

SUMMARY AND EXPLANATION

The Smart Detect™ SARS-CoV-2 rRT-PCR Kit is a molecular *in vitro* diagnostic assay that aids in the detection and diagnosis of COVID-19. The assay is based on widely used real-time reverse transcription polymerase chain reaction (rRT-PCR) technology, which employs oligonucleotide primers and probes labeled with fluorescent reporter dyes and quenchers. Viral RNA is first extracted from patient samples and then in the one-step rRT-PCR process, RNA is converted to cDNA. Next, the probes anneal to specific target sequences on the cDNA located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of the DNA polymerase degrades the probes, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. The Smart Detect™ SARS-CoV-2 rRT-PCR Kit utilizes four primer/probe sets. Different fluorescent reporter dyes/detection channels are assigned to each primer/probe set. Fluorescence intensity is monitored at each PCR cycle by 7500 Fast Dx Real-Time PCR instrument (Applied Biosystems) with Sequence Detection System (SDS) Software, version 1.4 (Applied Biosystems) or the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the CFX Maestro Software (Bio-Rad). The PCR cycle at which the fluorescence intensity surpasses a defined threshold value is used to determine results.

TEST PRINCIPLES

The Smart Detect™ SARS-CoV-2 rRT-PCR Kit is a real-time reverse transcription polymerase chain reaction (rRT-PCR) assay designed to qualitatively detect RNA from SARS-CoV-2 in respiratory specimens from individuals with signs and symptoms of infection who are suspected of COVID-19 [1]. Smart Detect™ SARS-CoV-2 rRT-PCR may be used with upper respiratory tract samples, such as nasopharyngeal swab, anterior nasal swab and mid-turbinate nasal swab samples. This test is a single-well multiplex one-step rRT-PCR assay for detection of three SARS-CoV-2 RNA targets: the envelope (E) gene, the nucleocapsid (N) gene, and a region of the open reading frame (ORF1b) of the RNA dependent RNA polymerase (RdRp) gene from the SARS-CoV-2 virus isolate Wuhan-Hu-1 (GenBank MN908947). The kit includes an Extraction Control (EC) and primers/probe designed to detect ribonuclease P (RNase P), a human housekeeping gene, in the EC and in extracted clinical specimens. Other control materials supplied with the kit include a Positive Control (PC) consisting of an RNA sequence containing all three SARS-CoV-2 target sequences and a No Template (Negative) Control (NTC) consisting of nuclease-free water. The Smart Detect™ SARS-CoV-2 rRT-PCR Kit is to be used with the 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems) or the CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

MATERIALS PROVIDED

The Smart *Detect*[™] SARS-CoV-2 rRT-PCR Kit contains the following components, which are sufficient for performing up to 48 rRT-PCR reactions.

1. **Positive Control (PC) (2 x 200 µL):** *in vitro* transcribed SARS-CoV-2 RNA in nuclease-free water. Store at -20°C or below.
2. **No Template Control (NTC) (1 x 1 mL):** Nuclease-free water. Store at -20°C or below.
3. **Extraction Control (EC) (2 x 1.2 mL):** Cultured HEK293 cells in DPBS buffer. Store at -20°C or below. Sufficient material to perform 16 extractions.
4. **Primer/Probe Mix (100 µM):** Vial containing all primers and probes in nuclease-free water (100 µM stock concentration). Store at -20°C or below in the dark. Sufficient material to perform 48 reactions.

Note: Additional copies of this Smart *Detect*[™] SARS-CoV-2 rRT-PCR Kit Package Insert can be obtained online at www.bit.ly/smartdetectpcrkit. Paper copies are available upon request at inquiries@inbios.com.

MATERIALS REQUIRED BUT NOT PROVIDED

RNA Extraction Kit	
Item	Vendor, catalog #
QIAamp Viral RNA Mini Kit	QIAGEN, 52904 or 52906

rRT-PCR Master Mix		
	Item	Vendor, catalog #
Option 1	Master Mix Component A	InBios, 500763
	Master Mix Component B	InBios, 201012
	Master Mix Component C	InBios, 201013
Option 2	Master Mix Component A	InBios, 500763
	Luna [®] Probe One-Step Reaction Mix (No ROX) (2X)	New England BioLabs, E3007E
	Luna [®] WarmStart [®] RT Enzyme Mix (20X)	
Option 3	TaqPath [™] 1-Step RT-qPCR Master Mix, CG	Life Technologies, A15299 and/or A15300

EQUIPMENT AND CONSUMABLES REQUIRED BUT NOT PROVIDED

Vortex
Microcentrifuge (with rotor for 1.5-2 mL tubes)
Bench top centrifuge (with rotor and adaptor for 96-well plates)
DNase/RNase free microcentrifuge tubes (1.5-2 mL)
Calibrated DNase/RNase-free pipettes of varying sizes (2 or 10 µL, 200 µL, 1000 µL)
DNase/RNase-free aerosol barrier pipette tips of varying sizes
Cold block(s) or ice
Nuclease-free water
Ethanol (96-100%)
Ethanol (70%)
10% bleach
DNAZap™ PCR DNA Degradation Solutions (Ambion, catalog # AM9890) or equivalent
RNase AWAY™ Decontamination Reagent (Invitrogen, catalog # 10328011) or equivalent
Disposable powder-free gloves
AND
7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems)
Sequence Detection System (SDS) Software, version 1.4 (Applied Biosystems)
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (Applied Biosystems, catalog # 4346906 or 4366932)*
MicroAmp™ Optical Adhesive Film (Applied Biosystems, catalog # 4360954 or 4311971)*
MicroAmp™ Fast 8-Tube Strips, 0.1 mL (Applied Biosystems, catalog # 4358293)*
MicroAmp™ Optical 8-Cap Strips (Applied Biosystems, catalog # 4323032)*
OR

CFX96 Touch Real-Time PCR Detection System (Bio-Rad)
CFX Maestro Software (Bio-Rad)
Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted (Bio-Rad, catalog # HSP9601, HSP9611, HSP9621, HSP9631, or HSP9641)*
Microseal® 'B' Adhesive Seals (Bio-Rad, catalog # MSB1001)*
0.2 ml 8-Tube PCR Strips without Caps, low profile, clear (Bio-Rad, catalog # TLS0801)*
0.2 ml Flat PCR Tube 8-Cap Strips, optical, ultraclear (Bio-Rad, catalog # TCS0803)*

*Either 8-tube PCR strips or 96-well PCR plates may be used.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic (IVD) use under Emergency Use Authorization (EUA) Only.
- Positive results are indicative of the presence of SARS-CoV-2 RNA.
- Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled in accordance with good laboratory procedure.
- For use only by personnel trained in the techniques of rRT-PCR and *in vitro* diagnostic procedures. A thorough understanding of the instructions for use is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following these instructions for use.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with COVID-19 is suspected, specimens should be collected with appropriate infection control precautions.
- For use only in specified laboratories by clinical laboratory personnel trained on the authorized rRT-PCR instrument.

Safety Precautions

- Handle all specimens as if infectious using safe laboratory procedures. Site- and activity-specific risk assessments should be performed to determine if enhanced biosafety precautions are warranted based on situational needs. Refer to the following CDC guidelines: Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons Under Investigation for Coronavirus Disease 2019 (<https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html>).
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses where reagents or human specimens are being handled.
- Do not pipette by mouth.
- Safety Data Sheets are available upon request.
- Manipulation of potentially infected specimens should be performed in a certified Class II BSC in a BSL-2 facility or higher. This includes aliquoting and/or diluting specimens and nucleic acid extraction procedures involving potentially infected specimens.
- Use appropriate personal protective equipment including but not limited to disposable gloves, laboratory coat/gown, and eye protection when handling specimens, reagents, pipettes, and other equipment.

Technical Precautions

- Use only the reagents described in the instructions for use. Do not use reagents from other manufacturers.
- Do not use expired reagents.
- Avoid contamination of clinical specimens or reagents with previously amplified PCR products. Assay workflow should entail separate working areas for specimen preparation, reaction set-up, and amplification/detection. Wear disposable gloves and change them before entering a separate working area.
- Maintain dedicated supplies and equipment for each of the separate working areas.
- Use a clean lab coat and clean disposable gloves for reaction set-up.
- Change gloves between samples and whenever contamination is suspected.
- Always use DNase/RNase-free aerosol barrier pipette tips. Use a fresh DNase/RNase-free aerosol barrier pipette tip for each reagent or specimen transfer.
- Avoid DNase/RNase contamination of the specimens. Decontaminate work surfaces, pipettes, and centrifuges with 10% bleach, DNAZap™ PCR DNA Degradation Solutions, and/or RNase AWAY™ Decontamination Reagent. Remove residual bleach with 70% ethanol.
- Avoid contamination by keeping reagent and reaction tubes capped or covered as much as possible.
- Do not open sealed PCR plates or PCR tubes after amplification to avoid contamination with amplified PCR products.
- Keep extracted RNA on cold block or on ice during reaction set-up.

SPECIMEN COLLECTION, HANDLING, STORAGE

- See CLSI MM13-A Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods, 1st Edition for information on specimen collection.
- For information on specimen collection for COVID-19, refer to the following CDC guidelines: Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons Under Investigation for Coronavirus Disease 2019 (<https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html>).
- Clinical specimens can be stored at 2-8°C for up to 72 hours after collection.
- If a delay in RNA extraction is expected, store specimens at -70°C or lower.

TEST PROCEDURE

Preparation of Positive Control (PC)

1. Store unused PC tubes provided with the kit at -20°C or below. Avoid freeze-thaw cycles.
2. Prior to use, thaw a single tube of PC. Mix gently by low speed vortex for 30 seconds, followed by centrifugation at 750 x g for 10 seconds.
3. Prepare single-use aliquots by aliquoting 25 µL of PC into 8 DNase/RNase-free microcentrifuge tubes.
4. Keep one single-use aliquot on cold block or ice until adding to the PCR reaction. After addition to PCR reaction, discard unused portion of the aliquot.
5. Store 7 remaining single-use aliquots at -20°C or below until use. Avoid freeze-thaw cycles.
6. If all 7 single-use aliquots are used, then repeat steps 2-5 with an unused PC tube.

Preparation of Extraction Control (EC)

1. Store unused EC tubes provided with the kit at -20°C or below. Avoid freeze-thaw cycles.
2. Prior to use, thaw a single tube of EC. Mix gently by low speed vortex for 30 seconds, followed by centrifugation at 750 x g for 10 seconds.
3. Prepare single-use aliquots by aliquoting 150 µL of EC into 8 DNase/RNase-free microcentrifuge tubes.
4. Keep one single-use aliquot on cold block or ice until RNA extraction. After RNA extraction, discard unused portion of the aliquot.
5. Store 7 remaining single-use aliquots at -20°C or below until use. Avoid freeze-thaw cycles.
6. If all 7 single-use aliquots are used, then repeat steps 2-5 with an unused EC tube.

Preparation of No Template Control (NTC)

1. Thaw the NTC (nuclease-free water) tube prior to use.
2. Aliquot into small volumes. **Note:** it is recommended to prepare single-use aliquots by aliquoting 25 µL of NTC in DNase/RNase-free microcentrifuge tubes.
3. Store single-use aliquots of NTC at -20°C or below until use.

RNA Extraction Procedure

The Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit must only be used with RNA extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, catalog # 52904 or 52906). Other RNA extraction kits have not been qualified or validated.

Extraction of EC must be performed with each batch of sample extractions to serve as an extraction control.

For each extraction batch:

1. Equilibrate samples to room temperature (15°C to 25°C).
2. Equilibrate a single-use aliquot of EC to room temperature (15°C to 25°C).
3. **Note:** Do not filter or centrifuge clinical specimens prior to RNA extraction. Do not filter EC prior to RNA extraction.
4. Perform the RNA extraction on the samples and EC following the manufacturer's instructions for use (Qiagen). The recommended starting volume of samples and EC is 140 µL. The recommended elution volume of samples and EC is 60 µL.
5. Keep extracted samples and EC on cold block or ice until adding to the PCR reaction.
6. After addition of extracted samples to the PCR reaction, store according to the manufacturer's instructions for viral RNA (Qiagen).

rRT-PCR Master Mix Option 1 or Option 2: Reaction Mixture Set-up

1. Thaw Master Mix Components A, B and C or Master Mix Component A, Luna[®] Probe One-Step Reaction Mix (No ROX) (2X), and Luna[®] WarmStart[®] RT Enzyme Mix (20X) at room temperature, then place on ice. After thawing, briefly mix each component by inversion, pipetting, or gentle vortexing.
2. Thaw the Primer/Probe Mix (100 µM) on cold block or ice. After thawing, vortex the Primer/Probe Mix briefly to mix, then centrifuge to collect the contents at the bottom of the tube.
3. Calculate the total volume required of each reaction component by multiplying the volume per reaction displayed in **Table 1** by the total number of reactions. **Note:** Include at least 10% excess volume of each component to compensate for volume loss during pipetting.

Table 1. Volume of each component per reaction

Component	Volume per reaction
Master Mix Component A	1 μ L
Master Mix Component B or Luna® Probe One-Step Reaction Mix (No ROX) (2X)	10 μ L
Master Mix Component C or Luna® WarmStart® RT Enzyme Mix (20X)	1 μ L
Primer/Probe Mix (100 μ M)	1 μ L
Nuclease-free water	2 μ L
Total volume per reaction	15 μ L

- Combine the required volume of each reaction component into a DNase/RNase free microcentrifuge tube to create the reaction mixture.
- Proceed to plate set-up.

rRT-PCR Master Mix Option 1 or Option 2: Plate Set-up

Keep the 96-well PCR plate or 8-tube PCR strip(s) covered as much as possible during addition of reaction mixture, controls, and samples. Change gloves often and when necessary to avoid contamination.

Reaction mixture and NTC

- Mix the reaction mixture thoroughly by gently pipetting or vortexing. Centrifuge briefly to collect liquid at the bottom of the tube.
- Add 15 μ L of the reaction mixture to the appropriate number of wells. Use consistent and accurate pipetting and minimize bubbles.
- Add 5 μ L of the NTC into the NTC well. An NTC must be included in each PCR run.
- Cover the 96-well PCR plate or 8-tube PCR strips and move to the specimen nucleic acid handling area to continue plating.

Samples, EC, and PC

- Mix the samples, EC, and PC by gentle inversion or flicking 3-5 times, then briefly centrifuge to collect the contents at the bottom of the tube.
- Add 5 μ L of each sample and EC into the appropriate PCR wells. **Figure 1** shows an example 96-well PCR plate layout. **Figure 2** shows an example 8-tube PCR strip layout.
- Lastly, add 5 μ L of the PC into the PC well. A PC must be included in each PCR run.
- If using a 96-well PCR plate, cover and seal the plate with an optical adhesive film. Centrifuge the plate briefly at 1,000 x g for 1 minute to collect the contents at the bottom of the wells and eliminate air bubbles.
- If using an 8-tube PCR strip, cap the strip with an optical 8-cap strip. Label the tabs on the end of the strip to indicate sample position. **Note:** Do not label the tops of the reaction tubes. Centrifuge the strip at 1,000 x g for 1 minute to collect the contents at the bottom of the wells and eliminate air bubbles.
- Keep the 96-well PCR plate or 8-tube PCR strip(s) in the dark until loading into the PCR instrument

rRT-PCR Master Mix Option 3: Reaction Mixture Set-up

- Thaw the TaqPath™ 1-Step RT-qPCR Master Mix on cold block or ice. After thawing, vortex the TaqPath™ 1-Step RT-qPCR Master Mix briefly to mix, then centrifuge to collect the contents at the bottom of the tube.
- Thaw the Primer/Probe Mix (100 μ M) on cold block or ice. After thawing, vortex the Primer/Probe Mix briefly to mix, then centrifuge to collect the contents at the bottom of the tube.
- Calculate the total volume required of each reaction component by multiplying the volume per reaction displayed in **Table 2** by the total number of reactions. **Note:** Include at least 10% excess volume of each component to compensate for volume loss during pipetting.

Table 2. Volume of each component per reaction

Component	Volume per reaction
4X TaqPath™ 1-Step RT-qPCR Master Mix	5 μ L
Nuclease-free water	9 μ L
Primer/Probe Mix (100 μ M)	1 μ L
Total volume per reaction	15 μ L

- Combine the required volume of each reaction component into a DNase/RNase free microcentrifuge tube to create the reaction mixture.
- Keep the microcentrifuge tube containing the reaction mixture on cold block or ice and proceed to plate set-up.

rRT-PCR Master Mix Option 3: Plate Set-up

Keep the 96-well PCR plate or 8-tube PCR strip(s) on cold block or ice during plating. Keep the plate covered as much as possible during addition of reaction mixture, controls, and samples. Change gloves often and when necessary to avoid contamination.

Reaction mixture and NTC

1. Mix the reaction mixture gently by low speed vortex for 30 seconds.
2. Add 15 µL of the reaction mixture to the appropriate number of wells. Use consistent and accurate pipetting and minimize bubbles.
3. Add 5 µL of the NTC into the NTC well. An NTC must be included on each 96-well PCR plate or in each set of 8-tube PCR strips.
4. Cover the 96-well PCR plate or 8-tube PCR strips and move to the specimen nucleic acid handling area to continue plating.

Samples, EC, and PC

1. Mix the samples, EC, and PC by gentle inversion or flicking 3-5 times, then briefly centrifuge to collect the contents at the bottom of the tube.
2. Add 5 µL of each sample and EC into the appropriate PCR wells. **Figure 1** shows an example 96-well PCR plate layout. **Figure 2** shows an example 8-tube PCR strip layout.
3. Lastly, add 5 µL of the PC into the PC well. A PC must be included on each 96-well PCR plate or in each set of 8-tube PCR strips.
4. If using a 96-well PCR plate, cover and seal the plate with an optical adhesive film. Vortex or invert the plate 3-5 times to mix the contents of the wells and then centrifuge at 150 x g for 1 minute to collect the contents at the bottom of the wells and eliminate air bubbles.
5. If using an 8-tube PCR strip, cap the strip with an optical 8-cap strip. Label the tabs on the end of the strip to indicate sample position. **Note:** Do not label the tops of the reaction tubes. Vortex or invert the strip 3-5 times to mix the contents of the wells and then centrifuge 150 x g for 1 minute to collect the contents at the bottom of the wells and eliminate air bubbles.
6. Keep the 96-well PCR plate or 8-tube PCR strip(s) on cold block or ice in the dark until loading into the PCR instrument.

Figure 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	EC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
B	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22
C	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34
D	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	PC
E												
F												
G												
H												

Figure 1. Example 96-well PCR plate layout for placement of 45 samples, 1 EC, 1 PC, and 1 NTC. S1, sample 1; EC, Extraction Control; PC, Positive Control; NTC, No Template Control**Figure 2**

1	2	3	4	5	6	7	8
NTC	EC	S1	S2	S3	S4	S5	PC

Figure 2. Example 8-tube PCR strip layout for placement of 5 samples, 1 EC, 1 PC, and 1 NTC. S1, sample 1; EC, Extraction Control; PC, Positive Control; NTC, No Template Control

Programming the 7500 Fast Dx Real-Time PCR Instrument

1. Turn on the 7500 Fast Dx Real-Time PCR Instrument.
2. Launch the SDS Software, version 1.4.
3. After a new window appears, select "Create New Document" from the menu.
4. The "New Document Wizard" screen will appear. Select the following parameters:
 - a. Assay: Standard Curve (Absolute Quantification)
 - b. Container: 96-well Clear
 - c. Template: Blank Document
 - d. Run Mode: Standard 7500
 - e. Operator: [Operator Name]
 - f. Comments: [Operator Comments]
 - g. Plate name: [Plate name]
5. After selecting the parameters and naming the plate, click "Next".
6. The "Select Detectors" screen will appear.
7. All four fluorescent detectors (assay targets) must be entered individually.
8. Click the "New Detector" button.
9. The "New Detector" window will appear.
10. Create the N detector as follows:
 - a. Name: N
 - b. Description: [Optional description (i.e. SARS-CoV-2 N target)]
 - c. Reporter Dye: Cy5

Note: The dyes that appear in the Reporter Dye list are those that have been previously entered using the Dye Manager. If a dye does not appear in the list, use the Dye Manager to add the dye and then return to this step in the procedure. Refer to the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument Technical Support or Online Help for more information.

- d. Quencher: (none)
- e. Color: Select a unique color for the detector as follows:
 - i. Click on the color rectangle to reveal the color options.
 - ii. Select a color by clicking on one of the color rectangles.
 - iii. Click "Ok" to return to "New Detector" window.
- f. Click "Ok" on the "New Detector" window to return to the "Select Detectors" screen.

11. Repeat steps 8-10 for each detector (assay target). The detector name, reporter dye, and quencher for each assay target are shown in the **Table 3**.

Table 3. List of detectors for the 7500 Fast Dx Real-Time PCR Instrument

Detector Name	Reporter Dye	Quencher Dye
N	Cy5	(none)
E	Texas Red	(none)
ORF1b	FAM	(none)
RNase P	VIC	(none)

12. After entering all four detectors, the “Detector Name”, “Description”, “Reporter”, and “Quencher” will appear in the “Select Detectors” screen on the left side of the screen.
13. All four detectors must be added to the “Detectors in Document” on the right side of the screen by clicking “Add >>”. After adding the four detectors to the document, the name of each detector should appear on the right side of the screen.
14. Once all detectors have been added to the document, select (none) for Passive Reference dye from the drop-down menu on the top right of the “Select Detectors” screen.
15. Click “Next” at the bottom of the “Select Detectors” window to proceed to “Set Up Sample Plate” window.
16. In the “Set Up Sample Plate” window, all four detectors will be assigned to each well to be tested in the assay.
17. Select all wells to be tested in the assay by dragging the cursor across the appropriate wells in plate map in the lower half of the screen.
18. In the upper half of the screen, select all four detectors by putting a check mark in the “Use” column. A colored “U” icon representing each detector will populate the wells.
19. Select “Finish” after all four detectors have been assigned to all wells.
20. After clicking “Finish”, the 7500 Fast Dx Real-Time PCR Instrument will initialize. The initialization is followed by a clicking noise. **Note:** The instrument must be turned on to initialize.
21. After initialization, the “Plate” tab will appear.
22. Each well of the plate to be tested should be populated with colored “U” icons corresponding to the detectors that were selected. To check detector assignments, select “Tools” from the file menu, then select “Detection Manager”.
23. The “Detector Manager” window will appear. Ensure that each detector is listed with the appropriate Reporter, Quencher, and Color selected.
24. If all detectors are present, select “Done”.
25. After creating and assigning detectors, select the “Instrument” tab.
26. If using rRT-PCR Master Mix Option 1 or Option 2, then program the thermal cycling conditions as shown in **Table 4** and follow steps 27-31 to complete instrument programming.
27. Under “Settings”, enter 20 µL for Sample Volume.
28. Under “Settings”, select Standard 7500 for Run Mode.
29. Under “Data Collection”, select Stage 3, Step 2 (60°C @ 0:60).
30. After programming the thermal cycling conditions and settings in the “Instrument” tab, the template file may be saved. To save the template file, select “File” from the menu and then select “Save As”.
31. Save the template file (.sdt) with the desired file name (i.e. Smart Detect SARS-CoV-2.sdt) in the desired file location.

Table 4. Thermal cycling conditions for the 7500 Fast Dx Real-Time PCR Instrument using rRT-PCR Master Mix Option 1 or Option 2

Stage	Step	Number of cycles	Temperature	Time
1	Reverse transcription	1	55°C	10 minutes
2	Initial denaturation	1	95°C	1 minute
3 (Step 1)	Denaturation	45	95°C	10 seconds
3 (Step 2)	Extension		60°C	60 seconds

32. If using rRT-PCR Master Mix Option 3, then program the thermal cycling conditions as shown in **Table 5** and follow steps 33-37 to complete instrument programming.
33. Under “Settings”, enter 20 µL for Sample Volume.
34. Under “Settings”, select Standard 7500 for Run Mode.
35. Under “Data Collection”, select Stage 4, Step 2 (58°C @ 0:30).
36. After programming the thermal cycling conditions and settings in the “Instrument” tab, the template file may be saved. To save the template file, select “File” from the menu and then select “Save As”.
37. Save the template file (.sdt) with the desired file name (i.e. Smart Detect SARS-CoV-2.sdt) in the desired file location.

Table 5. Thermal cycling conditions for the 7500 Fast Dx Real-Time PCR Instrument using rRT-PCR Master Mix Option 3

Stage	Step	Number of cycles	Temperature	Time
1	UNG incubation	1	25°C	2 minutes
2	RT incubation	1	50°C	15 minutes
3	Enzyme activation	1	95°C	2 minutes
4 (Step 1)	Amplification	45	95°C	3 seconds
4 (Step 2)			58°C	30 seconds

Running the 7500 Fast Dx Real-Time PCR Instrument

1. Turn on the 7500 Fast Dx Real-Time PCR Instrument.
2. Launch the SDS Software, version 1.4.
3. After the new window appears, select “Open Existing Document” from the menu.
4. Navigate to the saved location of the template file created in the previous section (i.e. Smart Detect SARS-CoV-2.sdt).
5. Double-click on the template file to open.
6. The 7500 Fast Dx Real-Time PCR Instrument will begin to initialize.
7. After initialization, the plate map will appear.
8. Click on the “Well Inspector” icon from the top menus.
9. Type sample identifiers for each well (sample IDs, EC, PC, NTC) into the Sample Name box in the “Well Inspector” window.
10. Once all sample and control identifiers are added, click the “Close” button on the “Well Inspector” window and return to the plate map.
11. Click the “Instrument” tab at the upper left corner.
12. The reaction conditions, volumes, and type of reaction will already be loaded based on the template file. Ensure settings are correct by referring to the “Programming the 7500 Fast Dx Real-Time PCR Instrument” section.
13. Before starting the run, the run file (.sds) must be saved. From the main menu, select “File”, then “Save As”. Designate a name for the file and save in an appropriate location.
14. If using a 96-well PCR plate, load the plate into plate holder of the 7500 Fast Dx Real-Time PCR Instrument. Ensure the plate is properly oriented with well A1 at the top left.
15. If using 8-tube PCR strip(s), load the strips vertically. If <12 strips are being run, center the strips in the middle of the plate holder and include “dummy” strips in columns 1 and 12 to ensure proper sealing of the block.
16. Once the run file is saved and the plate is loaded, click the “Start” button.

Data analysis for the 7500 Fast Dx Real-Time PCR Instrument

1. After the run has completed, select the “Results” tab at the upper left corner of the software.
2. Select the “Amplification Plot” tab to view the raw data.
3. Select all of the samples included in the assay run. This can be accomplished by clicking on the small box in the upper left-hand corner of each of the sample wells. The amplification curves should appear on the graph.
4. At the top right of the screen in the Data drop-down menu, select the Delta Rn vs. Cycle view.
5. The analysis settings for each detector must be selected individually.
6. Select “N” from the Detector drop-down menu.
7. Under “Analysis Settings”, select “Manual Ct” and “Manual Baseline” by clicking on the radio buttons. Set Manual Baseline as follows: Start (cycle): 5; End (cycle): 15.
8. The threshold must be set manually by dragging the red threshold line in the graph. The threshold should be adjusted to fall within the exponential phase of the fluorescence curves and above any background signal. The procedure used for setting the threshold should be used consistently. **Note:** See the “Troubleshooting” section for assistance.
9. Click the “Analyze” button. The red threshold line will turn green after analysis.
10. Repeat steps 6-9 for each detector (assay target).
11. Save the analysis file by selecting “File” then “Save As”. Designate a name for the file and save in an appropriate location.
12. Select the “Report” tab to display the cycle threshold (Ct) values.
13. Manually inspect amplification curves for all samples assigned a Ct value to verify positive amplification.
14. Proceed to the “Interpretation of Results” section for information on how to interpret Ct values.

Programming the CFX96 Touch Real-Time PCR Detection System

1. Turn on the CFX96 Touch Real-Time PCR Detection System.
2. Open the CFX Maestro Software. The software opens to the Home window and displays the Startup Wizard dialog box.

- On the Run setup tab of the Startup Wizard, select the instrument (CFX96) from the drop-down menu and click on User-defined as the run type.
- The Run Setup dialog box opens to the Protocol tab and displays the default protocol file. Click Create New in the upper left corner and the Protocol Editor will appear.
- Use the Protocol Editor to edit the default protocol. **Note:** the left pane of the Protocol Editor window contains the controls used to edit protocols.
- If using rRT-PCR Master Mix Option 1 or 2, then program the thermal cycling conditions and plate read step as shown in **Table 6** and follow steps 8-23 to complete instrument programming.

Table 6. Thermal cycling conditions and plate read steps for the CFX96 Touch Real-Time PCR Detection System using rRT-PCR Master Mix Option 1 or Option 2

Step	Description	Number of cycles	Temperature	Time
1	Reverse transcription	1	55°C	10 minutes
2	Initial denaturation	1	95°C	1 minute
3	Denaturation	1	95°C	10 seconds
4	Extension + Plate Read	1	60°C	30 seconds
5	GOTO 3 44 more times	44	N/A	N/A

- If using rRT-PCR Master Mix Option 3, then program the thermal cycling conditions and plate read step as shown in **Table 7** and follow steps 8-23 to complete instrument programming.

Table 7. Thermal cycling conditions and plate read steps for the CFX96 Touch Real-Time PCR Detection System using rRT-PCR Master Mix Option 3

Step	Description	Number of cycles	Temperature	Time
1	UNG incubation	1	25°C	2 minutes
2	RT incubation	1	50°C	15 minutes
3	Enzyme activation	1	95°C	2 minutes
4	Amplification	1	95°C	3 seconds
5	Amplification + Plate Read	1	58°C	30 seconds
6	GOTO 4 44 more times	44	N/A	N/A

- Set Sample Volume to 20 µL.
- Click OK to save the protocol and return to the Protocol tab in Run Setup.
- Review the details of the protocol. If correct, click Next to proceed to the Plate tab.
- On the Plate tab, click Create New and the Plate Editor appears.
- Use the Plate Editor to create a new plate.
- To ensure the correct the plate size is selected, go to Settings > Plate Size and check that 96-well is selected from the drop-down menu.
- To set the plate type, select Settings > Plate Type and select BR Clear from the drop-down menu.
- To set the scan mode, select the All Channels scan mode from the Scan Mode drop-down list in the Plate Editor toolbar.
- The assay targets and corresponding fluorophores for the CFX96 Touch are shown in **Table 8**.

Table 8. Assay targets and corresponding fluorophores for the CFX96 Touch Real-Time PCR Detection System

Assay Target	Reporter Dye
N	Cy5
E	Tex 615
ORF1b	FAM
RNase P	HEX

- To select fluorophores, click Select Fluorophores in the right pane. Select the following fluorophores by clicking the respective checkbox:
 - Channel 1: FAM
 - Channel 2: HEX
 - Channel 3: Tex 615
 - Channel 4: Cy5
 - Channel 5: none selected

Note: to change the display color of the fluorophore, click its Color box. The color will represent the fluorophore in the Plate Editor window and Data Analysis charts.

- Click OK to save changes and close the Select Fluorophores dialog box. When prompted, specify a name for the protocol and a save location.

19. The plate pane displays the plate map. Load the appropriate sample type to each well by selecting the well and selecting the appropriate Sample Type (Unknown, NTC, or Positive Control) from the drop-down menu. Multiple wells can be selected at once to load the sample type. **Note:** the EC can be listed as an Unknown sample.
20. In the Target Names section in the right pane, assign all four fluorophores (FAM, HEX, Tex 615, and Cy5) to each well by selecting the Load checkbox for each fluorophore. All wells can be selected at once to load the fluorophores.
21. Name each well as desired by typing in the sample name and pressing Enter in the Sample Names drop-down list in the right pane.
22. Click OK to save the plate and return to the Plate tab in Run Setup. When prompted, specify a name for the plate and a save location.
23. Review the details of the plate. If correct, proceed to the Start Run tab.

Running the CFX96 Touch Real-Time PCR Detection System

1. In the Start Run tab, review the protocol and plate details in the Run Information section.
2. Select the checkbox for the appropriate block (CFX96) on which to perform the run.
3. To insert the plate or 8-tube strips into the block, click Open Lid.
4. Insert the plate or 8-tube strips into the block. Ensure the plate or 8-tube strips are properly oriented.
5. Click Close Lid.
6. Click Start Run at the bottom right of the screen.
7. When prompted, save the data file (.pcrd).

Data analysis for the CFX96 Touch Real-Time PCR Detection System

1. After the run has completed, open the data file (.pcrd) by going to Select File > Open > Data File in the Home window and locating the desired data file. Adjust the following settings as described below.
2. Select Settings > Cq Determination mode and select Single Threshold. In Single Threshold mode, adjust the threshold for each fluorophore by clicking on the threshold line in the Amplification chart and moving the line to fall within the exponential phase of the fluorescence curves and above any background signal. The procedure used for the setting the threshold should be used consistently. **Note:** See the "Troubleshooting" section for assistance.
3. Select Settings > Baseline Setting and select Baseline Subtracted.
4. Select Settings > Analysis Mode and select analysis by fluorophore.
5. Select Settings > Cycles to Analyze and the Cycles to Analyze dialog box appears. Enter the starting cycle as 6 and ending cycle as 45 for all targets and click OK.
6. Cq values of each well are displayed in the Quantification Data tab.
7. Manually inspect amplification curves for all samples assigned a Cq value to verify positive amplification. Amplification curves are displayed in the Quantification tab.
8. Proceed to the "Interpretation of Results" section for information on how to interpret Cq values. **Note:** Cq values are equivalent to Ct values in the "Interpretation of Results" section.

Disposal

1. Do not open sealed PCR plates or PCR tubes after amplification to avoid contamination with amplified PCR products.
2. Dispose of hazardous or biologically contaminated materials according to institutional practices.

QUALITY CONTROL CRITERIA

- Quality control requirements must be performed in accordance with local, state and federal regulations.
- Always include an NTC and PC in each rRT-PCR run.
- Always include an EC in each sample extraction batch and in subsequent rRT-PCR runs.

INTERPRETATION OF RESULTS

Controls

1. The NTC should test negative and not demonstrate any amplification above the threshold (i.e. should not exceed background levels) in any detection channel.
2. The PC should test positive and should demonstrate amplification below the designated Ct cutoff (< 40) in the same detection channels as the target primer/probes.
3. The EC should test positive (i.e. amplification below the designated Ct cutoff (< 40)) in the RNase P detection channel.
4. **Table 9** shows the expected performance of the controls in the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit.
5. The results for the assay controls (PC, NTC, and EC) should be examined prior to interpretation of clinical specimen results. If the controls are not valid, the clinical specimen results cannot be interpreted, and the assay should be repeated.

Table 9. Expected performance of the controls in the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit

Control	N	E	ORF1b	RNase P	Expected Ct values
Positive Control (PC)*	+	+	+	-	< 40
No Template Control (NTC)*	-	-	-	-	None detected
Extraction Control (EC)**	-	-	-	+	< 40

*If the PC or NTC deviates from the expected Ct values, then the assay results are invalid and the rRT-PCR reaction should be repeated.

**If the EC deviates from the expected Ct values, then the assay results are invalid and the extraction procedure and rRT-PCR reaction should be repeated.

Interpretation of clinical specimen results

1. Assessment of clinical specimen test results should be performed after the PC, NTC, and EC have been examined and determined to be valid and acceptable. If the controls are not valid, the clinical specimen results cannot be interpreted.
2. To interpret clinical specimen results, the Ct cutoff value is 40. A Ct value < 40 indicates that the specimen contains the target nucleic acid sequence or RNase P sequence and is positive (+). A Ct value ≥ 40 indicates that the specimen does not contain the target nucleic acid sequence or RNase P sequence and is negative (-). Interpretation of clinical specimen results based on all three targets and RNase P is shown in **Table 10**.

Table 10. Interpretation of clinical specimen results

Target gene/region Ct < 40 = positive (+) Ct value ≥ 40 = negative (-)				Final Interpretation of Clinical Specimen Results
N	E	ORF1b	RNase P	
-	-	-	+	SARS-CoV-2 negative
+	+	+	+	SARS-CoV-2 positive
+	+	-	+	SARS-CoV-2 positive
+	-	+	+	SARS-CoV-2 positive
-	+	+	+	SARS-CoV-2 positive
+	-	-	+	SARS-CoV-2 presumptive positive; retest specimen*
-	-	+	+	SARS-CoV-2 presumptive positive; retest specimen*
-	+	-	+	SARS-CoV-2 presumptive positive; retest specimen*
+	+	+	-	SARS-CoV-2 positive
+	+	-	-	SARS-CoV-2 positive
+	-	+	-	SARS-CoV-2 positive
-	+	+	-	SARS-CoV-2 positive
+	-	-	-	SARS-CoV-2 presumptive positive; retest specimen*
-	+	-	-	SARS-CoV-2 presumptive positive; retest specimen*
-	-	+	-	SARS-CoV-2 presumptive positive; retest specimen*
-	-	-	-	Invalid; retest specimen**

*Result for SARS-CoV-2 RNA is Presumptive Positive. Sample should be retested by re-extracting RNA from the same specimen. For samples with a repeated Presumptive Positive result, additional confirmatory testing may be conducted, if it is necessary to differentiate between SARS-CoV-2 and SARS-CoV-1 or other Sarbecovirus currently unknown to infect humans, for epidemiological purposes or clinical management.

**First conduct the repeat test by re-extracting RNA from the same specimen. If the test fails again, collect a new specimen from the patient and repeat the test.

TROUBLESHOOTING

Positive Control (PC)

Problem	Potential causes and solutions
PC is negative for N, E, and/or ORF1b* <i>*If the PC deviates from expected performance (Table 9), then the assay results are invalid and the rRT-PCR reaction should be repeated.</i>	Incorrect storage of PC <ul style="list-style-type: none"> • Store at -20°C or below. • Prepare and use single-use aliquots. • Avoid freeze-thaw cycles. Pipetting error during plating (incorrect well or incorrect volume) <ul style="list-style-type: none"> • Ensure 5 µL of PC is added to 15 µL of the reaction mixture. Inadequate mixing of PCR reagents <ul style="list-style-type: none"> • Ensure the contents of wells are thoroughly mixed according to the plating instructions. Expired and/or contaminated reagent(s) <ul style="list-style-type: none"> • Check expiration dates of PC, Primer/Probe Mix, master mix, and nuclease-free water. Incorrect PCR instrument settings <ul style="list-style-type: none"> • Ensure correct fluorophores are assigned to targets (see Table 3 for 7500 Fast Dx Real-Time PCR Instrument and Table 8 for CFX96 Touch Real-Time Detection System).

Extraction Control (EC)

Problem	Potential causes and solutions
EC is negative for RNase P* <i>*If the EC deviates from expected performance (Table 9), then the assay results are invalid and the extraction procedure and rRT-PCR reaction should be repeated.</i>	Incorrect storage of EC <ul style="list-style-type: none"> • Store at -20°C or below. • Prepare and use single-use aliquots. • Avoid freeze-thaw cycles. Extraction error <ul style="list-style-type: none"> • Ensure 140 µL of EC is used in extraction. • Follow manufacturer's instructions for use for extraction kit (Qiagen, catalog # 52904 or 52906) EC filtered before extraction <ul style="list-style-type: none"> • Do not filter EC before extraction because this will reduce the amount of cellular material present for extraction. Pipetting error during plating (incorrect well or incorrect volume) <ul style="list-style-type: none"> • Ensure 5 µL of extracted EC is added to 15 µL of the reaction mixture. Inadequate mixing of PCR reagents <ul style="list-style-type: none"> • Ensure the contents of wells are thoroughly mixed according to the plating instructions. Expired and/or contaminated reagent(s) <ul style="list-style-type: none"> • Check expiration dates of EC, Primer/Probe Mix, and master mix, and nuclease-free water. Incorrect PCR instrument settings <ul style="list-style-type: none"> • Ensure correct fluorophores is assigned to RNase P (VIC on 7500 Fast Dx Real-Time PCR Instrument and HEX on CFX96 Touch Real-Time PCR Detection System).

No Template Control (NTC)

Problem	Potential causes and solutions
<p>Amplification in the NTC*</p> <p><i>*If the NTC deviates from expected performance (Table 9), then the assay results are invalid and the rRT-PCR reaction should be repeated.</i></p>	<p>Contamination</p> <ul style="list-style-type: none"> • Ensure proper decontamination of work surfaces. • Use a clean lab coat and clean disposable gloves for reaction set-up. • Use aerosol barrier pipette tips. • Prepare and use single-use aliquots. • Keep the contents of wells covered as much as possible during and after plating. • Ensure PCR plates and strips are fully sealed before placing in the PCR instrument. <p>Pipetting error during plating (incorrect well or incorrect volume)</p> <ul style="list-style-type: none"> • Ensure 5 µL NTC is added to 15 µL of the reaction mixture.

RNase P

Problem	Potential causes and solutions
<p>Clinical specimen is negative for RNase P (human house-keeping gene/internal control)*</p> <p><i>*See Table 10. If a clinical specimen is negative for all SARS-CoV-2 targets and RNase P, then the result is "invalid; retest specimen". First conduct a repeat test by re-extracting RNA from the same specimen. If the test fails again, collect a new specimen from the patient and repeat the test.</i></p>	<p>Poor specimen quality (insufficient host cells/genetic material)</p> <ul style="list-style-type: none"> • Collect new clinical specimen. <p>Incorrect storage of clinical specimen/RNA degradation</p> <ul style="list-style-type: none"> • Store at 2-8°C for up to 72 hours after collection. • If a delay in RNA extraction is expected, store at -70°C or lower. <p>Extraction error</p> <ul style="list-style-type: none"> • Ensure 140 µL of clinical specimen is used in extraction. • Follow manufacturer's instructions for use for extraction kit (Qiagen, catalog # 52904 or 52906) <p>Clinical specimen filtered before extraction</p> <ul style="list-style-type: none"> • Do not filter specimen before extraction because filtering will reduce the amount of host cells/genetic material present for extraction. <p>Pipetting error during plating (incorrect well or incorrect volume)</p> <ul style="list-style-type: none"> • Ensure 5 µL of each extracted clinical specimen is added to 15 µL of the reaction mixture. <p>Inadequate mixing of PCR reagents</p> <ul style="list-style-type: none"> • Ensure the contents of wells are thoroughly mixed according to the plating instructions. <p>Incorrect PCR instrument settings</p> <ul style="list-style-type: none"> • Ensure correct fluorophore is assigned to RNase P (VIC on 7500 Fast Dx Real-time PCR Instrument and HEX on CFX96 Touch Real-Time PCR Detection System).

7500 Fast Dx Real-Time PCR Instrument

Problem	Potential causes and solutions
Difficulty setting threshold line for N, E, ORF1b, and/or RNase P	<p>Abnormal amplification curves</p> <ul style="list-style-type: none"> Ensure correct “Analysis Settings” are selected. For each detector (assay target), the threshold line should be placed above any background signal and within the exponential phase of the fluorescence curves. See Figure 3A-3D for examples of threshold placement for each assay target. After setting the threshold line, inspect all samples assigned a Ct value to verify positive amplification (i.e. a sigmoidal amplification curve) and adjust the threshold as needed. Bumpy or jagged amplification curves may indicate that viral RNA in the specimen is near the LoD of the assay. See Figure 4 for an example.
Obtaining very low Ct values (i.e. <5) in a well for a single assay target	<p>High background</p> <ul style="list-style-type: none"> Ensure that the threshold line is placed above any background signal and within the exponential phase of the fluorescence curves. See Figure 5 for an example of the background fluorescence that may be observed from NTCs. After setting the threshold line, inspect all samples assigned a Ct value to verify positive amplification (i.e. a sigmoidal amplification curve) and adjust the threshold as needed.

CFX96 Touch Real-Time PCR Detection System

Problem	Potential causes and solutions
Difficulty setting threshold line for N, E, ORF1b, and/or RNase P	<p>Abnormal amplification curves</p> <ul style="list-style-type: none"> Ensure correct Baseline Settings, Analysis Mode, and Cycles to Analyze are selected. For each assay target, the threshold line should be placed above any background signal and within the exponential phase of the fluorescence curves. See Figure 6A-6D for examples of threshold placement for each assay target. After setting the threshold line, inspect all samples assigned a Ct value to verify positive amplification (i.e. a sigmoidal curve). Bumpy or jagged amplification curves may indicate that the viral RNA in the specimen is near the LoD of the assay.

Figure 3A

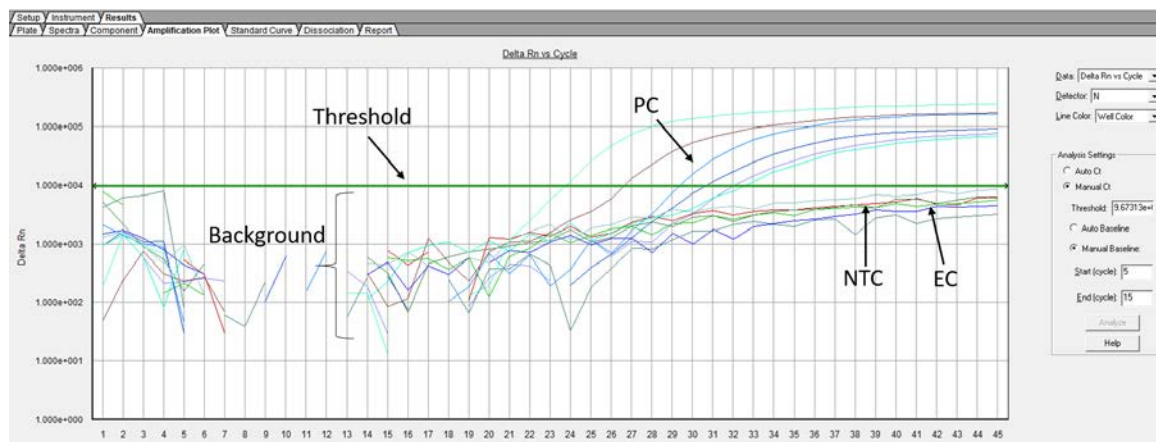


Figure 3B

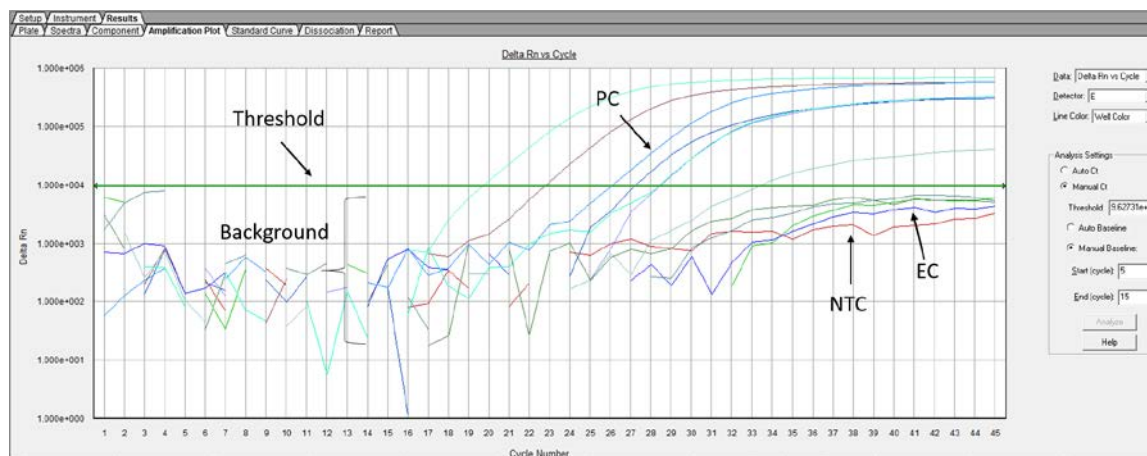


Figure 3C

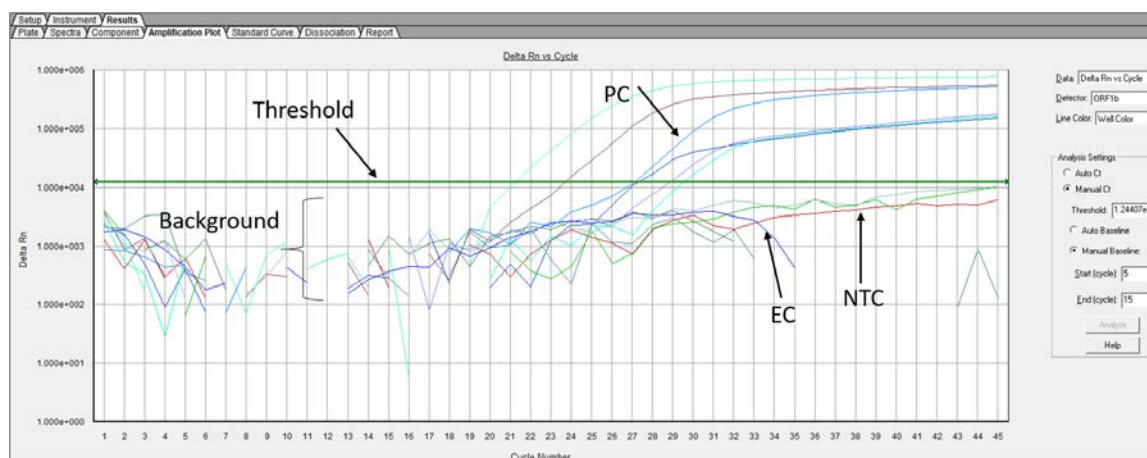


Figure 3D

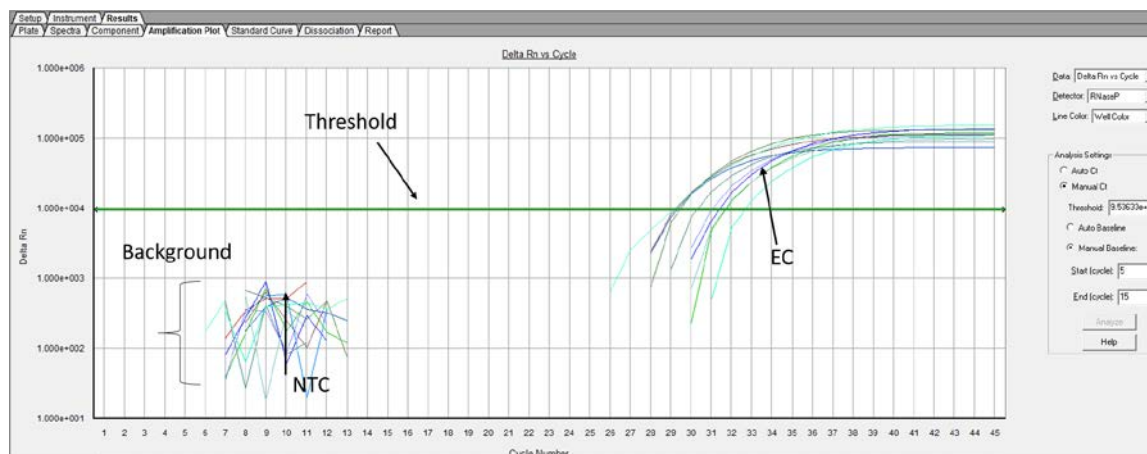


Figure 3A-3D. Amplification plots for the N (3A), E (3B), ORF1b (3C), and RNase P(3D) assay targets for clinical specimens run on the 7500 Fast Dx Real-Time PCR Instrument. For N, E, and ORF1b, the threshold line should be placed above the background signal (including valid NTC and EC signals) and within the exponential phase of the amplification curves. For RNase P, the threshold line should be placed above the background signal (including valid NTC signal) and within the exponential phase of the amplification curves. EC, extraction control; NTC, no template control; PC, positive control

Figure 4

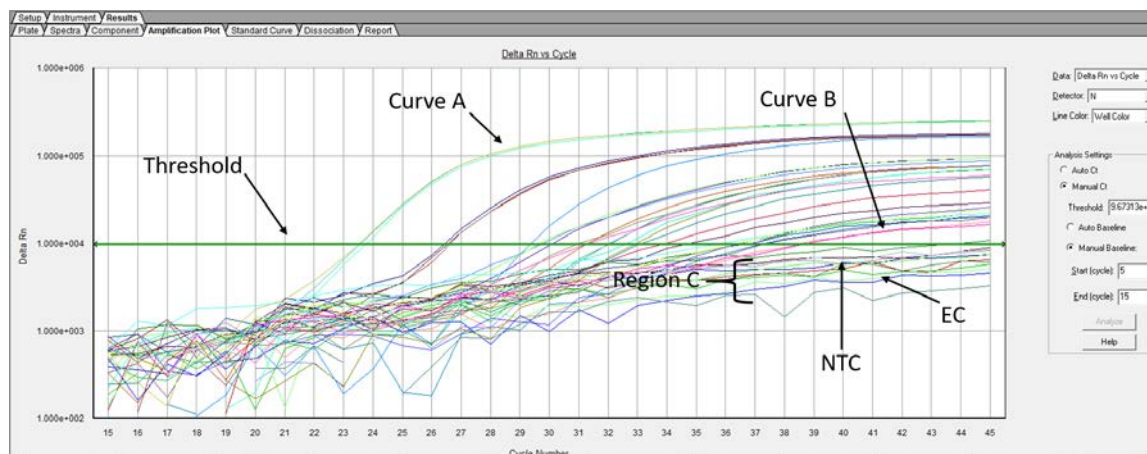


Figure 4. Amplification plot for the N assay target for clinical specimens run on the 7500 Fast Dx Real-Time PCR Instrument. Curve A depicts a smooth, sigmoidal amplification curve with a strong fluorescent signal. Curve B depicts a smooth, sigmoidal amplification curve with a weaker fluorescent signal than curve A. Region C depicts several bumpy/jagged amplification curves, which do not resemble the positive amplification curves of Curve A and Curve B. The threshold line should be placed above the background signal (including valid NTC and EC signals) and within the exponential phase of the amplification curves demonstrating smooth, sigmoidal amplification curves. EC, extraction control; NTC, no template control

Figure 5

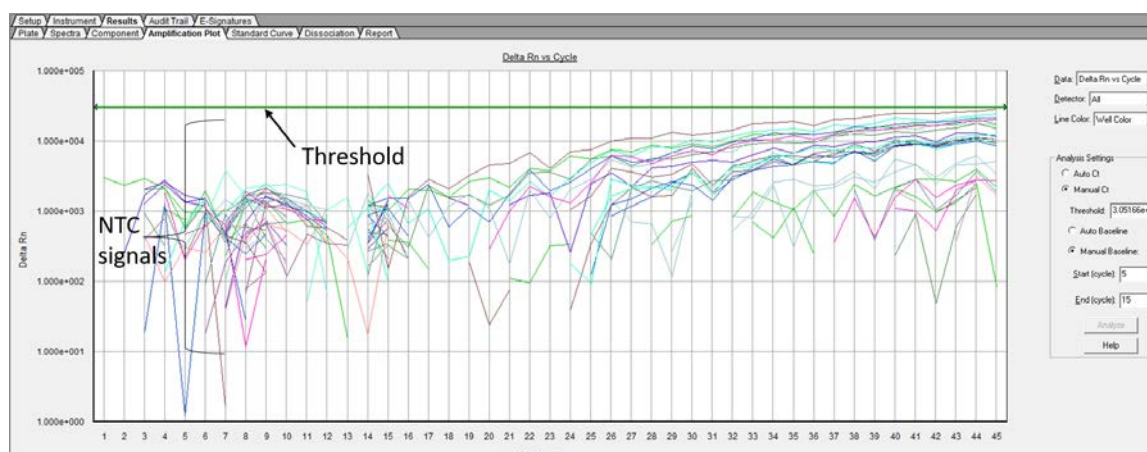


Figure 5. Amplification plot for all assay targets for 20 NTCs run on the 7500 Fast Dx Real-Time PCR Instrument demonstrating typical range of background signal derived from NTCs. NTC, no template control

Figure 6A

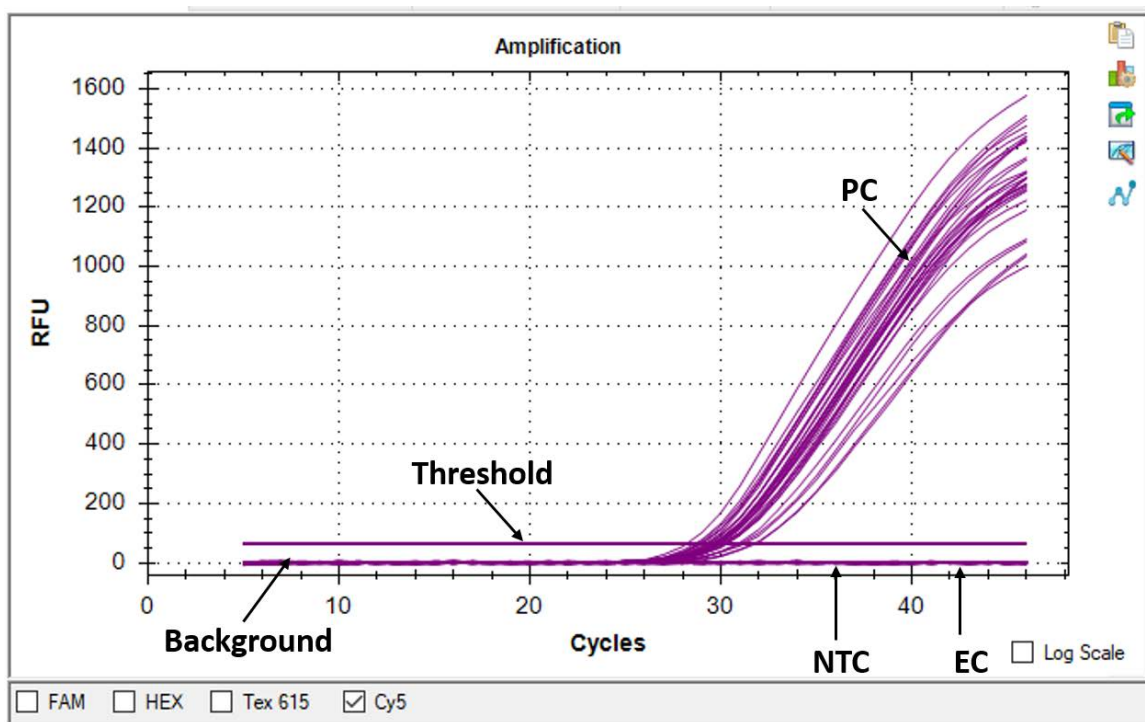


Figure 6B

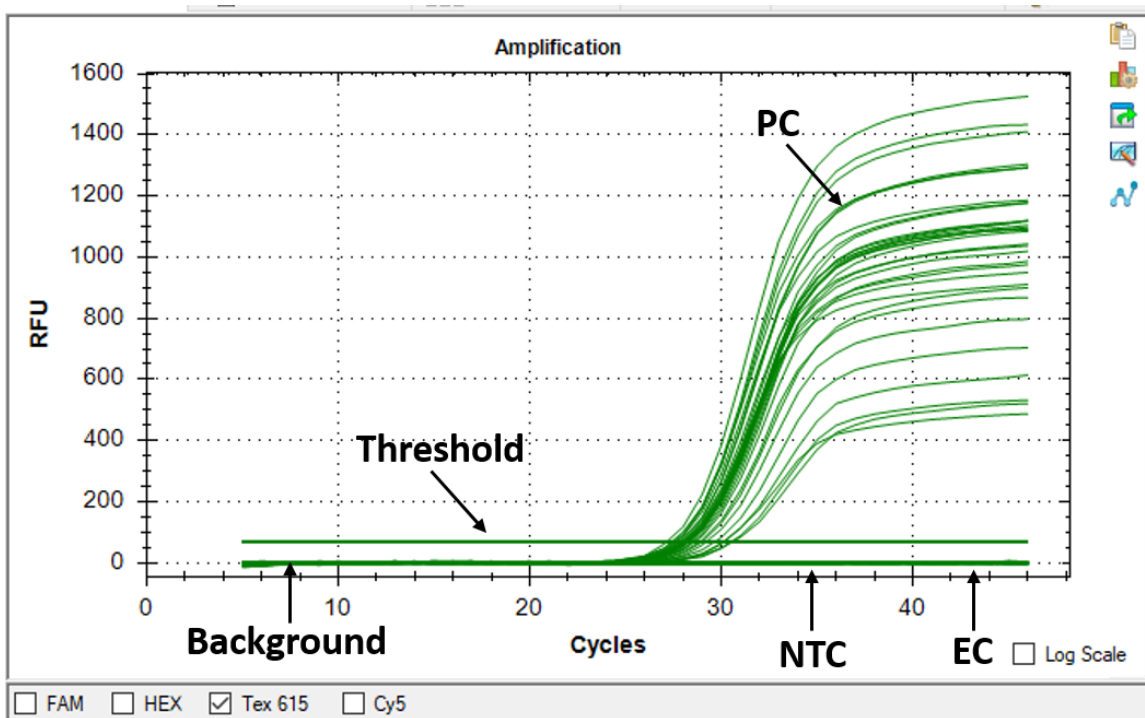


Figure 6C

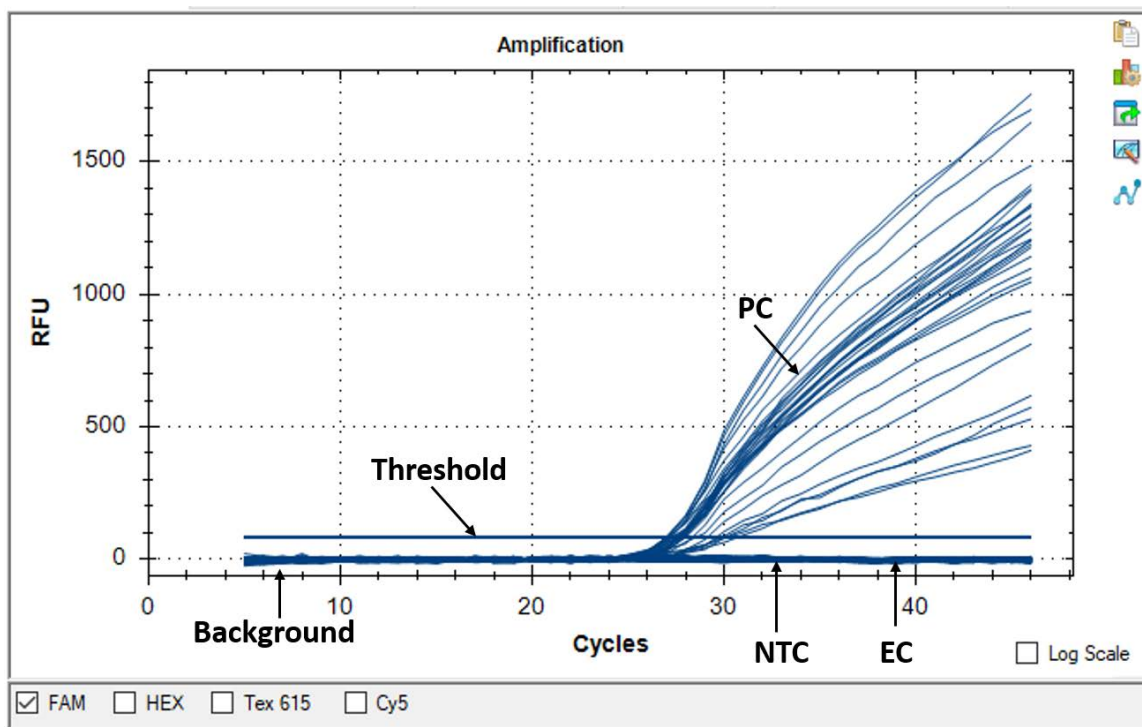


Figure 6D

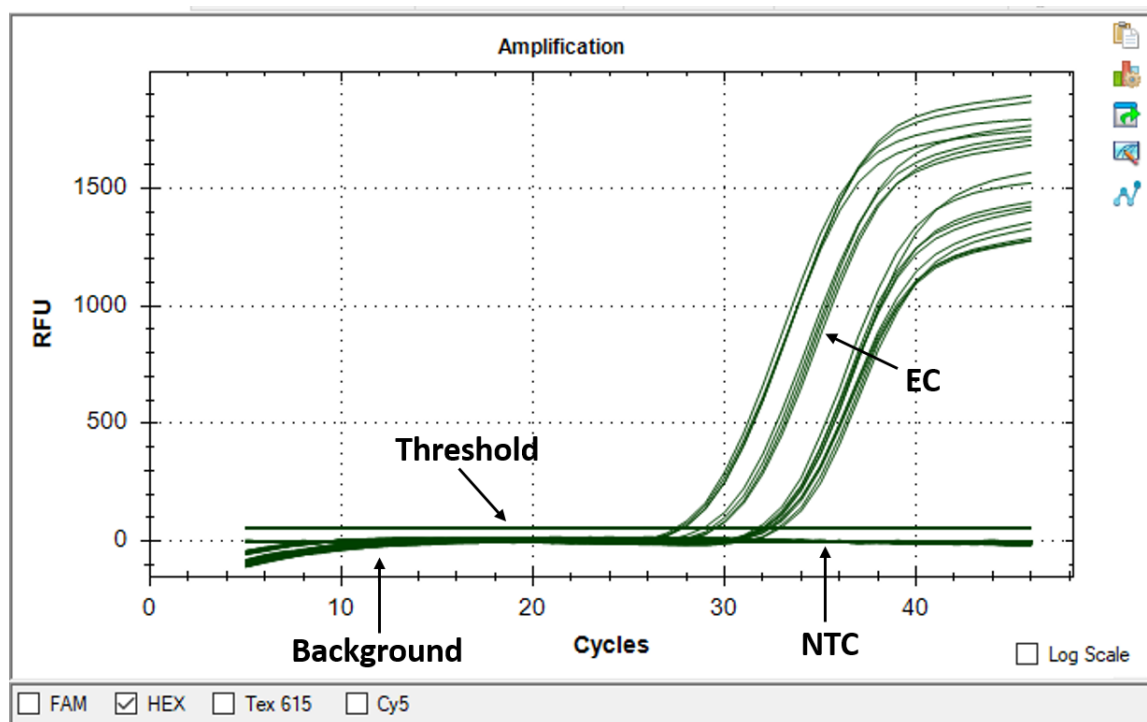


Figure 6A-6D. Amplification plots for the N (6A), E (6B), ORF1b (6C), and RNase P (6D) assay targets for clinical specimens run on the CFX96 Touch Real-Time PCR Detection System. For N, E, and ORF1b, the threshold line should be placed above the background signal (including valid NTC and EC signals) and within the exponential phase of the amplification curves. For RNase P, the threshold line should be placed above the background signal (including valid NTC signal) and within the exponential phase of the amplification curves. EC, extraction control; NTC, no template control; PC, positive control

LIMITATIONS

- For use only by personnel trained in the techniques of rRT-PCR and *in vitro* diagnostic procedures. A thorough understanding of the instructions for use is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following these instructions for use.
- The performance of Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was established using nasopharyngeal swab samples. Anterior nasal swabs and mid-turbinate nasal swabs are also considered acceptable specimen types for use with the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit but performance has not been established. Testing of nasal and mid-turbinate nasal swabs (self-collected or collected by a healthcare provider) is limited to patients with symptoms of COVID-19. Please refer to FDA's FAQs on Diagnostic Testing for SARS-CoV-2 for additional information.
- Based on the *in silico* analysis, SARS-CoV-1 and other SARS-like coronaviruses in the same subgenus (Sarbecovirus) as SARS-CoV-2 may cross-react with the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit. SARS-CoV-1 is not known to be currently circulating in the human population, therefore is highly unlikely to be present in patient specimens.
- SARS-CoV-2 may mutate in one, two, or three of the target regions of the Smart *Detect*TM SARS-CoV-2 rRT-PCR assay. If this occurs, then SARS-CoV-2 may not be detected.
- Test performance is affected by the clinical specimen quality originally obtained by the clinician.
- False negative results may arise from improper specimen handling and degradation of the viral RNA during shipping/storage.
- False positive results may happen from cross-contamination between patient samples, specimen mix-up and RNA contamination during product handling.
- Detection of SARS-CoV-2 RNA indicates presence of viral RNA, however this does not confirm that SARS-CoV-2 is the causative agent of clinical symptoms.
- The Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit does not rule out diseases caused by other bacterial or viral pathogens.

CONDITIONS OF AUTHORIZATION FOR THE LABORATORY

The Smart *Detect*TM SARS-CoV-2 rRT-PCR 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/MedicalDevices/Safety/EmergencySituations/ucm161496.htm>.

However, to assist clinical laboratories using the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit ("your product" in the conditions below), the relevant Conditions of Authorization are listed below:

- Authorized laboratories¹ using the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit will include with result reports of your product, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories using Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit will use the product as outlined in the Instructions for Use. 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 the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit are not permitted.
- Authorized laboratories that receive the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit will notify the relevant public health authorities of their intent to run the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit prior to initiating testing.
- Authorized laboratories using the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories will collect information on the performance of the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and InBios International, Inc. (covid19EUA@inbios.com) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit of which they become aware.
- All laboratory personnel using the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit and use the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit in accordance with the authorized labeling.
- InBios International, Inc., authorized distributors, and authorized laboratories using the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

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

PERFORMANCE CHARACTERISTICS

The performance of the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was evaluated by the following analytical studies:

ANALYTICAL SENSITIVITY (LIMIT OF DETECTION)

Limit of detection (LoD) studies determine the lowest detectable concentration of SARS-CoV-2 RNA at which greater than or equal to 95% of all replicates test positive.

To determine LoD for the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit, serial dilutions of SARS-CoV-2 whole viral genomic RNA were prepared by spiking into pooled nasopharyngeal swab matrix. The SARS-CoV-2 whole viral genomic RNA used in this study was sourced from BEI (catalog no. NR-52285, lot 70033320) and was determined to contain 4.8E+07 genome equivalents (GE)/mL by Bio-Rad QX200 Droplet Digital PCR System.

Initial LoD was determined by testing 3-fold serial dilutions in triplicate. Each replicate was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, catalog # 52904 or 52906) and tested on the 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems). The lowest concentration at which all three replicates tested positive was treated as the initial LoD.

To establish final LoD, 20 replicates spiked with SARS-CoV-2 whole viral genomic RNA at the initial LoD concentration were tested. Testing of 20 replicates was repeated at increasing concentrations until at least 95% positivity was achieved. Both 6.9 GE/reaction (6.0E+02 GE/mL) and 10 GE/reaction (8.6E+02 GE/mL) resulted in a <95% positivity rate. A 95% positivity rate was achieved with 12.5 GE/reaction (1.1E+03 GE/mL), therefore the final LoD for the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was determined to be 12.5 GE/reaction or 1.1E+03 GE/mL on the 7500 Fast Dx Real-Time PCR Instrument (**Table 11**).

Table 11. Final LoD determination on the 7500 Fast Dx Real-Time PCR Instrument

LoD (SARS-CoV-2 GE/mL)	LoD (SARS-CoV-2 GE/reaction)	# Positive (Ct <40) / # Replicates Tested			# Positive (by Final Results Interpretation) / # Replicates Tested	Positivity Rate (%)
		N	E	ORF1b		
6.0E+02	6.9	13/20	19/20	0/20	13/20	65%
8.6E+02	10	17/20	20/20	0/20	17/20	85%
1.1E+03	12.5	19/20	20/20	0/20	19/20	95%

Alternate Instrument Testing

An abbreviated LoD study was conducted to determine the LoD for the Smart *Detect*TM rRT-PCR Kit using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

LoD for the CFX96 Touch Real-Time PCR Detection System was evaluated by testing 20 replicates of pooled nasopharyngeal swab matrix spiked with SARS-CoV-2 whole viral genomic RNA sourced from BEI at the initial LoD concentration determined in the LoD study for the 7500 Fast Dx Real-Time PCR Instrument. Each replicate was extracted using QIAamp Viral RNA Mini Kit and tested on the CFX96 Touch Real-Time PCR Detection System. Testing of 20 replicates was repeated at increasing concentrations until at least 95% positivity was achieved. 6.9 GE/reaction (6.0E+02 GE/mL) resulted in a <95% positivity rate. A 95% positivity rate was achieved with 10 GE/reaction (8.6E+02 GE/mL). The final LoD for the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was determined to be 10 GE/reaction or 8.6E+02 GE/mL on the CFX96 Touch Real-Time PCR Detection System (**Table 12**).

Table 12. Final LoD determination on the CFX96 Touch Real-Time PCR Detection System

LoD (SARS-CoV-2 GE/mL)	LoD (SARS-CoV-2 GE/reaction)	# Positive (Ct <40) / # Replicates Tested			# Positive (by Final Results Interpretation) / # Replicates Tested	Positivity Rate (%)
		N	E	ORF1b		
6.0E+02	6.9	15/20	20/20	0/20	15/20	75%
8.6E+02	10	19/20	20/20	0/20	19/20	95%

Alternate Master Mix Testing

An equivalency study was conducted to evaluate the performance of alternate rRT-PCR master mixes with the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit.

InBios Master Mix Component A, B, and C (rRT-PCR Master Mix Option 1) and InBios Master Mix Component A, Luna® Probe One-Step Reaction Mix (No ROX) (2X) and Luna® WarmStart® RT Enzyme Mix (20X) (rRT-PCR Master Mix Option 2) were evaluated for equivalency as compared to the TaqPath™ 1-Step RT-qPCR Master Mix, CG (rRT-PCR Master Mix Option 3), the master mix used in the analytical sensitivity (LoD) study.

Equivalency was evaluated using an abbreviated LoD study. Twenty replicates of pooled nasopharyngeal swab matrix were spiked with SARS-CoV-2 whole viral genomic RNA sourced from BEI at 12.5 GE/reaction (1.1E+03 GE/mL), the final LoD determined for the 7500 Fast Dx Real-Time PCR Instrument using rRT-PCR Master Mix Option 3. Each replicate was extracted using QIAamp Viral RNA Mini Kit. Replicates were tested on the 7500 Fast Dx Real-Time PCR Instrument and the CFX96 Touch Real-Time PCR Detection System using rRT-PCR Master Mix Option 1 and Option 2. Testing of twenty replicates was repeated at decreasing concentrations until at least 95% positivity was achieved.

The final LoD for the Smart Detect™ SARS-CoV-2 rRT-PCR Kit using rRT-PCR Master Mix Option 1 and Option 2 was determined to be 12.5 GE/reaction (1.1E+03 GE/mL) on the 7500 Fast Dx Real-Time PCR Instrument, therefore rRT-PCR Master Mix Option 1 and Option 2 demonstrated equivalent performance compared with rRT-PCR Master Mix Option 3 on the 7500 Fast Dx Real-Time PCR Instrument (**Table 13**).

The final LoD for the Smart Detect™ SARS-CoV-2 rRT-PCR Kit using rRT-PCR Master Mix Option 1 and Option 2 was determined to be 8 GE/reaction (6.9E+02 GE/mL) on the CFX96 Touch Real-Time PCR Detection System, therefore rRT-PCR Master Mix Option 1 and Option 2 demonstrated improved performance (<1x LoD) compared with rRT-PCR Master Mix Option 3 on the CFX96 Touch Real-Time PCR Detection System (**Table 13**).

Table 13. Comparison of master mix LoDs for each PCR instrument

Instrument	rRT-PCR Master Mix Option 1 and 2	rRT-PCR Master Mix Option 3
	LoD GE/reaction (GE/mL)	LoD GE/reaction (GE/mL)
7500 Fast Dx Real-Time PCR Instrument	12.5 (1.1E+03)	12.5 (1.1E+03)
CFX96 Touch Real-Time PCR Detection System	8 (6.9E+02)	10 (8.6E+02)

ANALYTICAL REACTIVITY (INCLUSIVITY)

The inclusivity of the Smart Detect™ SARS-CoV-2 rRT-PCR Kit was evaluated by *in silico* analysis. Smart Detect™ primer and probe sequences were queried individually against 156 complete genome sequences of SARS-CoV-2 deposited in the NCBI database as of March 26, 2020.

The N primer and probe sequences demonstrated 100% identity with all but one SARS-CoV-2 sequence. For this one sequence (GenBank MT246485.1) a single nucleotide mismatch was identified in the middle of the binding region of the reverse primer (alignment of 19/20). This single SARS-CoV-2 mutation is not predicted to impact reverse primer binding or assay performance.

The E primer and probe sequences demonstrated 100% identity with all but one SARS-CoV-2 sequence. Upon further analysis of the subject sequence, one single nucleotide mismatch was found in the 3' end of the E probe binding region resulting in an alignment of 25/26 bases. This single SARS-CoV-2 mutation is not anticipated to affect probe binding or assay performance.

The ORF1b primer and probe sequences demonstrated 100% identity to all SARS-CoV-2 sequences.

In conclusion, Smart Detect™ SARS-CoV-2 rRT-PCR Kit primers and probes are highly conserved to the complete genome sequences of SARS-CoV-2 in the NCBI database as of March 26, 2020.

ANALYTICAL SPECIFICITY (CROSS-REACTIVITY)

Cross-reactivity of the Smart Detect™ SARS-CoV-2 rRT-PCR Kit was evaluated by *in silico* analysis and by wet testing whole organisms and purified nucleic acids from pathogens potentially found in upper respiratory specimens.

For the *in silico* analysis, the Smart Detect™ SARS-CoV-2 rRT-PCR Kit primer and probe sequences were queried individually against sequences of 30 pathogens potentially present in upper respiratory specimens and/or with genetic similarities to SARS-CoV-2. FDA defines *in silico* cross-reactivity as greater than 80% identity between one of the primers/probes and any sequence present in the targeted pathogen. *in silico* results demonstrate potential cross-reactivity of N, E, and ORF1b primer/probe sequences with SARS coronavirus (**Table 14**). No cross-reactivity is expected with other human coronaviruses or other pathogens.

Table 14. Predicted cross-reactivity of Smart Detect™ primers/probes with various pathogens

Pathogen	Predicted cross-reactivity – % identity
----------	---

	N primer/probes	E primer/probes	ORF1b primer/probes
Human coronavirus 229E	None	None	None
Human coronavirus OC43	None	None	None
Human coronavirus HKU1	None	None	None
Human coronavirus NL63	None	None	None
SARS coronavirus	≥95%	100%	≥88%
MERS coronavirus	None	None	None
Adenovirus	None	None	None
Human Metapneumovirus (hMPV)	None	None	None
Human parainfluenza virus 1	None	None	None
Human parainfluenza virus 2	None	None	None
Human parainfluenza virus 3	None	None	None
Human parainfluenza virus 4	None	None	None
Influenza A	None	None	None
Influenza B	None	None	None
Enterovirus 68	None	None	None
Respiratory syncytial virus	None	None	None
Rhinovirus	None	None	None
<i>Chlamydia pneumoniae</i>	None	None	None
<i>Haemophilus influenzae</i>	None	None	None
<i>Legionella pneumophila</i>	None	None	None
<i>Mycobacterium tuberculosis</i>	None	None	None
<i>Streptococcus pneumoniae</i>	None	None	None
<i>Streptococcus pyogenes</i>	None	None	None
<i>Bordetella pertussis</i>	None	None	None
<i>Mycoplasma pneumoniae</i>	None	None	None
<i>Pneumocystis jirovecii</i> (PJP)	None	None	None
<i>Candida albicans</i>	None	None	None
<i>Pseudomonas aeruginosa</i>	None	None	None
<i>Staphylococcus epidermidis</i>	None	None	None
<i>Staphylococcus salivarius</i>	None	None	None

For wet testing, pathogens listed in **Table 15** were spiked into pooled nasopharyngeal or simulated nasopharyngeal swab matrix. Each spiked sample was extracted using the QIAamp Viral RNA Mini Kit and tested on the 7500 Fast Dx Real-Time PCR Instrument. All potential cross-reactive specimens tested negative with the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit, therefore no cross-reactivity was observed among the wet-tested pathogens.

Table 15. Wet testing results for potential cross-reactive pathogens

Pathogen/Strain	Matrix	Concentration	Final Results Interpretation
Human adenovirus 1 ATCC VR-1	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Human parainfluenza virus 1 ATCC VR-94	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Human parainfluenza virus 2 ATCC VR-92	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Parainfluenza virus 3 ATCC VR-1782	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Human rhinovirus 64 ATCC VR-1174	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Human respiratory syncytial virus ATCC VR-26	Simulated nasopharyngeal swab	1.0E+05 PFU/mL	Negative
Human respiratory syncytial virus (genomic RNA)	Pooled nasopharyngeal swab	1.79E+00 ng total RNA/ μ L	Negative
MERS-coronavirus (genomic RNA)	Pooled nasopharyngeal swab	4.30E-02 ng total RNA/ μ L	Negative
Influenza A (genomic RNA)	Pooled nasopharyngeal swab	3.50E-01 ng total RNA/ μ L	Negative
Influenza B (genomic RNA)	Pooled nasopharyngeal swab	7.47E+00 ng total RNA/ μ L	Negative
Enterovirus D68 (genomic RNA)	Pooled nasopharyngeal swab	9.00E-03 ng total RNA/ μ L	Negative
<i>Mycobacterium tuberculosis</i> (genomic DNA)	Pooled nasopharyngeal swab	1.38E+02 ng gDNA/ μ L	Negative
Human metapneumovirus (genomic RNA)	Pooled nasopharyngeal swab	2.59E-01 ng total RNA/ μ L	Negative
Human metapneumovirus ATCC [®] VR-3250SD TM (Quantitative Synthetic RNA)	Simulated nasopharyngeal swab	$\geq 1.0E+05$ copies/ μ L	Negative
Human bocavirus ATCC [®] VR-3251SD TM (Quantitative Synthetic DNA)	Simulated nasopharyngeal swab	$\geq 1.0E+05$ copies/ μ L	Negative

CLINICAL EVALUATION

The clinical performance of the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was determined by testing 30 contrived positive samples and 30 negative samples. Contrived positive samples were prepared by spiking SARS-CoV-2 viral genomic RNA into individual clinical nasopharyngeal swab samples collected from patients in the United States with signs and symptoms of respiratory infections and/or confirmed positive for respiratory infections other than SARS-CoV-2. Negative samples were the same individual clinical nasopharyngeal swab samples without any spiking. The SARS-CoV-2 whole viral genomic RNA used in this study was sourced from BEI (catalog no. NR-52285, lot 70033320). This study was performed at InBios International, Inc.

Each sample was extracted using QIAamp Viral RNA Mini Kit. Positive contrived samples were spiked with viral RNA after incubation of the sample with the extraction kit lysis buffer to preserve the integrity of the unprotected RNA. Following extraction, samples were randomized, blinded, and coded. All blinded samples were tested on the 7500 Fast Dx Real-Time PCR Instrument as per the product insert.

The positive percent agreement (PPA) for the 1x-2x LoD and 3x-5x LoD samples and the negative percent agreement (NPA) for the 0x LoD (negative) samples were determined by comparing the results obtained to the expected results. A summary of the PPA and NPA by concentration is presented in **Table 16**. The overall PPA for the positive samples tested with the Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit was 100% [95% CI: 88.4%-100%]. One false positive result was obtained out of 30 negative samples tested resulting in an NPA of 96.7% [95% CI: 82.8%-99.9%].

Table 16. Smart *Detect*TM SARS-CoV-2 rRT-PCR Kit % Agreement with Expected Results by Sample Concentration

SARS-CoV-2 concentration (LoD)	Results (# Positive /# Tested)	Average Ct N target	Average Ct E target	Average Ct ORF1b target	% Agreement [95% CIs]
1x-2x LoD	20/20	35.90	32.28	38.24	100% [83.2%-100%]*
3x-5x LoD	10/10	32.61	29.57	33.67	100% [69.2%-100%]*
0x LoD (negative)	1**/30	N/A	N/A	N/A	96.7% [82.8%-99.9%]

*Overall PPA for 1x-5x LoD: 100% (30/30) [95% CI: 88.4%-100%]

**One negative sample (collected in the United States on January 9, 2020) exhibited Ct values <40 for N and E genes. Upon closer inspection of the amplification curves after unblinding, both the N and E curves for this sample appear non-sigmoidal in shape and were incorrectly called positive.

REFERENCES

[1] Evaluating and Reporting Persons Under Investigation (PUI). *Coronavirus Disease 2019 (COVID-19)*. [Online] CDC, 2020. <https://www.cdc.gov/coronavirus/2019-ncov/hcp/clinical-criteria.html>.



InBios International, Inc.
307 Westlake Ave. N., Suite 300
Seattle, WA 98109 USA
1-866-INBIOS1 (USA, Toll Free)
1-206-344-5821 (International)

Technical Support: <https://inbios.com/technical-support/>

Insert Part No. 900251-02
 Catalog No. COV2-E
Effective Date: 07/01/2020
www.inbios.com