



BioGX

Molecular Made Easy

Xfree COVID-19 Direct RT-PCR

REF 500-003-XMP



104 Extraction-Free Sample-Direct Tests

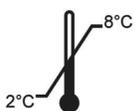
Instructions For Use

For *In Vitro* Diagnostic Use

For Emergency Use Authorization (EUA) Only

For use with:

Applied Biosystems QuantStudio™ 5 and 7500 Fast Dx
Bio-Rad CFX96 Touch™ and CFX384 Touch™



IVD

R_x Only

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INTENDED USE

The BioGX Xfree™ COVID-19 Direct RT-PCR (Xfree™ COVID-19) assay is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in nasopharyngeal, anterior nasal, mid-turbinate, and oropharyngeal swab specimens, nasopharyngeal wash/aspirate or nasal aspirates obtained 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. SARS-CoV-2 RNA is generally detectable in upper 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 the disease. Laboratories within the United States and its territories are required to report all 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 Xfree™ COVID-19 assay is intended for use by qualified clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR *in vitro* diagnostic procedures. The Xfree™ COVID-19 assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

SUMMARY AND EXPLANATION OF THE TEST

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the novel betacoronavirus that causes the COVID-19 respiratory disease. The COVID-19 respiratory disease is transmitted amongst infected humans within populations via respiratory droplets from talking, coughing, and sneezing^{1,2}. Symptoms of COVID-19 range from mild illness (dry cough, fatigue, fever, and shortness of breath) to severe illness and death¹.

COVID-19 was first identified on December 31, 2019, amongst patients suffering from pneumonia in Wuhan, China^{1,2}. SARS-CoV-2 is the seventh coronavirus identified as a human pathogen that likely evolved within an animal host or within humans following zoonotic transfer³.

As of September 29, 2020, the WHO has confirmed 33,034,598 infections and 996,342 COVID-19 related deaths globally². The elderly, immunocompromised, and those with cardiovascular disease, diabetes, and chronic respiratory disease have been shown to be at higher risk for severe illness^{1,2}. Ahead of global vaccination efforts, patient testing and social distancing measures mandated by world governments have proven to be the only way to stem transmission rates.

BioGX Xfree™ COVID-19 assay is a real-time RT-PCR test that utilizes primer and probe sequences based on the United States Centers for Disease Control and Prevention (US CDC) assay for detection of SARS-CoV-2 (N1 gene region) and human RNase P^{4,5,6}. Additionally, a non-naturally occurring single-stranded RNA has been integrated into the lyophilized format to serve as an internal amplification control.

The PCR assay is a multiplex test that utilizes a BioGX Sample-Ready™ lyophilized reagent provided in 2 mL vials. Each vial contains all PCR components such as primers, probes, enzymes, dNTPs, MgCl₂, Internal Amplification Control RNA (IAC), and buffers required for real-time RT-PCR-based testing. The assay has been validated utilizing two different workflows, the direct workflow in which patient sample is added directly to the reconstituted BioGX RT-PCR mix without any pre-processing or extraction steps, and the non-direct workflow that requires nucleic acid extraction using the QIAGEN QIAamp Viral RNA Mini Kit. Each lyophilized vial contains enough material to test 104 direct patient samples or 160 extracted/purified nucleic acid samples with one the following platforms:

- Applied Biosystems QuantStudio™ 5** utilizing Design & Analysis software version 1.5.1
- Applied Biosystems 7500 Fast Dx** utilizing SDS software version 1.4.1.
- Bio-Rad CFX96 Touch™** utilizing Maestro software version 1.1
- Bio-Rad CFX384 Touch™** utilizing Maestro software version 1.1

Applied Biosystems QuantStudio 5 trademark is the property of ThermoFisher Scientific.
Bio-Rad CFX96 Touch and Bio-Rad CFX384 Touch trademarks are the property of Bio-Rad Laboratories.

PRINCIPLES OF THE PROCEDURE

The BioGX Xfree™ COVID-19 assay is to be used with the Applied Biosystems QuantStudio™ 5, Applied Biosystems 7500 Fast Dx, Bio-Rad CFX96 Touch™ or Bio-Rad CFX384 Touch™ platforms with direct patient sample or extracted patient sample. The template RNA is reverse transcribed into cDNA and target sequences are PCR amplified. The amplified target(s) is (are) detected during amplification using hydrolysis probes labeled at one end with a fluorescent reporter dye (fluorophore) and at the other end with a quencher moiety. Probes labeled with different fluorophores are used to detect

specific amplicons originating from SARS CoV-2 (N1), RNase P, and Internal Amplification Control in the following optical channels:

- | | |
|----------------------------------|------------------------------|
| ● RNase P | FAM equivalent channel |
| ● unused | HEX equivalent channel |
| ● SARS-CoV-2 (N1) | Texas Red equivalent channel |
| ● Internal Amplification Control | Cy 5 equivalent channel |
| ● unused | Cy 5.5 equivalent channel |

When the probes are in their native state, the fluorescence of the fluorophore is quenched due to its proximity to the quencher. However, in the presence of their specific target cDNA, the probes hybridize to their complementary sequences and are then hydrolyzed by the 5'-3' exonuclease activity of the DNA polymerase as it synthesizes the nascent strand along the cDNA template. As a result, the fluorophores are separated from their quencher molecules and fluorescence is emitted. The Applied Biosystems QuantStudio™ 5, Applied Biosystems 7500 Fast Dx, Bio-Rad CFX96 Touch™ or Bio-Rad CFX384 Touch™ platforms measure these signals at the end of each amplification cycle in real time and interpret the data to provide a qualitative result for each of the above targets. A positive result for the detection of target RNA is indicated by the presence of a real-time PCR growth curve and an associated Ct (Cycle threshold) value.

REAGENTS REQUIRED (PROVIDED)

Qty	REF	Contents	No. Tests Direct Format	No. Tests Extracted Format
4 vials	500-003-XMP	BioGX Xfree™ COVID-19 Direct RT-PCR - Multi-Platform Sample-Ready™ lyophilized PCRMaster Mix containing polymerase, reverse transcriptase, primers, probes, dNTPs, MgCl ₂ , Internal Amplification Control RNA (IAC), and buffers	104	160

NOTE: Safety Data Sheets (SDS) are available at www.biogx.com or by request.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- RNA Control (External Control):
 - ZeptoMetrix (catalog number: NATSARS(CoV2)-ST) or
 - BioGX Synthetic Nucleocapsid Phosphoprotein Gene Region (N1) (catalog no. 720-0206)
- Sterile DNase/RNase free 1.5 mL snap cap tubes
- BioGX Molecular Grade Water or equivalent

- BioGX Rehydration Water Part number: 800-0035-12
- Sterile swab collection device appropriate for nasopharyngeal, anterior nasal, mid-turbinate or oropharyngeal swab collections
- Copan universal transport media (UTM®) collection kit
- Copan ESwab™ collection kit
- BD™ universal viral transport (UVT) collection kit
- Sterile viral transport media (VTM)
- Sterile saline (0.85% NaCl)
- Vortex Genie 2 Vortexer (VWR catalog no. 58815-234) or equivalent
- Disposable nitrile gloves
- Calibrated Manual or Repeater Pipettes capable of accurately dispensing: 2 µL, 10 µL, 100 µL, 1000 µL
- Sterile DNase/RNase free filter tip pipette tips
- Optional if using extraction: QIAGEN QIAamp Viral RNA Mini Kit (REF 52906)
 - Centrifuge compatible with QIAGEN extraction kit tubes
- Applied Biosystems QuantStudio 5 (0.2 mL) with Design & Analysis software v1.5.1 and consumables
 - Thermo Fisher optical 8-tube strip (catalog no. 4316567)
 - Thermo Fisher ultra-clear optical caps, strips of 8 (catalog no. AB-0866)
 - Thermo Fisher 96-well optical clear reaction plates (catalog no. A36924)
 - Thermo Fisher 96-well qPCR plate seals (catalog no. AB-1170)
- Applied Biosystems 7500 Fast Dx (0.1 mL) with SDS software v1.4.1 and consumables
 - Thermo Fisher optical 8-tube strip (catalog no. 4358293)
 - Thermo Fisher ultra-clear optical caps, strips of 8 (catalog no. 4323032)
 - Thermo Fisher 96-well optical reaction plates (catalog no. 4346906)
 - Thermo Fisher 96-well qPCR plate seals (catalog no. 4311971)
- Bio-Rad CFX96 Touch with Maestro software v1.1 and consumables
 - Bio-Rad 8-tube PCR strips without caps (catalog no. TLS0851)
 - Bio-Rad Optical flat 8-cap strips for PCR tubes (catalog no. TCS0803)
 - Bio-Rad 96-well plates (catalog no. HSP9655)
 - Bio-Rad 96-well plate sealing film, optical (catalog no. MSB1001)
- Bio-Rad CFX384 Touch with software v1.1 and consumables
 - Bio-Rad 384-well plates (catalog no. HSP3905)
 - Bio-Rad 384-well plate sealing film, optical (catalog no. MSB1001)

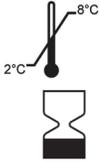
WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use.
- For prescription use only.
- For use under an Emergency Use Authorization (EUA) only.
- The Xfree™ COVID-19 assay has not been FDA cleared or approved, but has been authorized by FDA under an EUA for use by authorized laboratories; laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.
- The Xfree™ COVID-19 assay has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.
- 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.
- If infection with SARS-CoV-2 is suspected based on current clinical screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Specimen processing should be performed in accordance with national biological safety regulations.
- Treat all biological specimens as if capable of transmitting infectious agents in accordance with safe laboratory procedures such as those described in CLSI Document M29⁷ and in Biosafety in Microbiological and Biomedical Laboratories⁸.
- Performance characteristics of this test have been established only with the specimen types listed in “Intended Use” section. The performance of this assay with other specimen types or samples has not been evaluated.
- Do not use the reagents if the protective pouches are open or torn upon arrival.
- Close reagent protective pouches promptly with the zip seal after each use. Remove any excess air in the pouches prior to sealing and store at 2-8 °C.
- Do not remove desiccant from the PCR master mix pouches.
- Do not use master mix if the desiccant is not present or is broken inside the pouches.
- Do not use reagent vials if they are opened or damaged.
- Do not mix reagents from different pouches and/or kits and/or lots.
- Do not use expired reagents and/or materials.
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kits are being handled.
- Dispose of unused reagents and waste in accordance with country, federal, provincial, state, and local regulations.
- Clean and disinfect all surfaces with a 10% bleach solution followed by molecular grade water.



- Use clean gloves when handling PCR reagents.

REAGENT STORAGE AND STABILITY



- Reagents have been tested to demonstrate optimal performance when stored properly at 2-8°C and consumed by the expiration date on the kit label.
- Avoid exposing the reagents (lyophilized or rehydrated) to direct sunlight or long-term ambient lighting.
- **Discard unused rehydrated master mix if not used immediately. Re-use may result in contamination and false results.**
- **If** pouched is opened by the user but lyophilized reagent is not rehydrated, avoid exposure of lyophilized reagent to moisture.



SPECIMEN COLLECTION, STORAGE, AND HANDLING

Wear gloves when handling Copan Universal Transport Media (UTM), BD Universal Viral Transport (UVT), Copan ESwab™, or Saline collected specimens. If gloves come in contact with the specimen, immediately change gloves to prevent contamination of other specimens.

Collection: Nasopharyngeal/Oropharyngeal swab specimens should be collected according to the manufacturer's instructions for use. Swab specimens should be collected using only swabs with a synthetic tip, and an aluminum or plastic shaft. Calcium alginate swabs are not acceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing either 3 mL of viral transport media (UTM/VTM) or 1 mL of modified liquid Amies media (ESwab™).

Nasopharyngeal swab (NP): Insert a swab into nostril parallel to the palate. Swab should reach depth equal to distance from nostrils to outer opening of the ear. Leave swab in place for several seconds to absorb secretions. Slowly remove swab while rotating it.

Oropharyngeal swab (e.g., throat swab, OP): Swab the posterior pharynx, avoiding the tongue.

Anterior Nasal Swab (NS): Using a flocced or spun polyester swab, insert the swab at least 1 cm (0.5 inch) inside the nostril (naris) and firmly sample the nasal membrane by rotating the swab and leaving in place for 10 to 15 seconds. Sample both nostrils with same swab.

Storage: Specimens may be stored at 2-8°C for up to 72 hours after collection. If a delay in testing or shipping is expected, store specimens at -70°C or below.

Shipping: Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Store specimens at 2-8°C and ship overnight to the lab on ice pack. Specimens frozen at -70°C are shipped overnight to the lab on dry ice.

Additional information on packing, shipping, and transporting of specimens can be found at Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19).

For further guidance on specimen collection, storage and handling from patients suspected to be infected with SARS-CoV-2, reference the following CDC Interim Guidelines: <https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html>.

INSTRUCTIONS FOR USE

Instrument Templates/Protocols and Programming

Please refer to Appendix E for appropriate EUO labeling of instrument platforms.

Applied Biosystems QuantStudio™ 5

(Operating software: Design & Analysis Software v1.5.1)

Install the BioGX Protocol on the Applied Biosystems QuantStudio™ 5

It will be necessary to import the Applied Biosystems QuantStudio™ 5 template. The most current Applied Biosystems QuantStudio™ 5 template is available for download at www.biogx.com. Follow the 'Education Center' tab in the navigation menu at the top of the home page. Select "US Product Documents". Follow the "Xfree™ Direct RT-PCR Documents" tab. Choose the appropriate product number and follow the "Run Files" link to the platform of choice. Download the Applied Biosystems QuantStudio™ 5 template. Alternatively, the Applied Biosystems QuantStudio™ 5 template can also be obtained by emailing BioGX at covid19@biogx.com. To manually program the thermocycler, please refer to **Appendix A**. For further instrument information, please refer to the Applied Biosystems QuantStudio™ 5 user manual⁹.

Applied Biosystems 7500 Fast Dx

(Operating software: SDS Software v1.4.1)

Install the BioGX Protocol on the Applied Biosystems 7500 Fast Dx

It will be necessary to import the Applied Biosystems 7500 Fast Dx template. The most current Applied Biosystems 7500 Fast Dx template is available for download at www.biogx.com. Follow the “Education Center” tab in the navigation menu at the top of the home page. Select “US Product Documents”. Follow the “Xfree™ Direct RT-PCR Documents” tab. Choose the appropriate product number and follow the “Run Files” link to the platform of choice. Download the Applied Biosystems 7500 Fast Dx template. Alternatively, the Applied Biosystems 7500 Fast Dx template can also be obtained by emailing BioGX at covid19@biogx.com. To manually program the thermocycler, please refer to **Appendix B**. For further instrument information, please refer to the Applied Biosystems 7500 Fast Dx user manual¹⁰.

Bio-Rad CFX96 Touch™

(Operating software: Maestro™ 1.1)

Install the BioGX Protocol on the Bio-Rad CFX96 Touch™

It will be necessary to import the Bio-Rad CFX96 Touch™ Protocol and Plate files. The most current versions of the Bio-Rad CFX96 Touch™ Protocol and Plate files are available for download on www.biogx.com. Follow the “Education Center” tab in the navigation menu at the top of the home page. Select “US Product Documents”. Follow the “Xfree™ Direct RT-PCR Documents” tab. Choose the appropriate product number and follow the “Run Files” link to the platform of choice. Download the Bio-Rad CFX96 Touch™ Protocol and Plate files. Alternatively, the Bio-Rad CFX96 Touch™ Protocol and Plate files can also be obtained by emailing BioGX at covid19@biogx.com. To manually program the thermocycler, please refer to **Appendix C**. For further instrument information, please refer to the Bio-Rad CFX96 Touch™ user manual¹¹.

Bio-Rad CFX384 Touch™

(Operating software: Maestro™ 1.1)

Install the BioGX Protocol on the Bio-Rad CFX384 Touch™

It will be necessary to import the Bio-Rad CFX384 Touch™ Protocol and Plate files. The most current versions of the Bio-Rad CFX384 Touch™ Protocol and Plate files are available for download on www.biogx.com. Follow the “Education Center” tab in the navigation menu at the top of the home page. Select “US Product Documents”. Follow the “Xfree™ Direct RT-PCR Documents” tab. Choose the appropriate product number and follow the “Run Files” link to the platform of choice. Download the Bio-Rad CFX384 Touch™ Protocol and Plate files. Alternatively, the Bio-Rad CFX384 Touch™ Protocol and

Plate files can also be obtained by emailing BioGX at covid19@biogx.com. To manually program the thermocycler, please refer to **Appendix D**. For further instrument information, please refer to the Bio-Rad CFX384 Touch™ user manual¹¹.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE

Follow the “RT-PCR Set-up - Direct Patient Sample” section below for the appropriate real-time PCR platform (i.e. QuantStudio™ 5, 7500 Fast Dx, CFX96 Touch™ and CFX384 Touch™).

Applied Biosystems QuantStudio™ 5



1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 26 samples to be tested by direct sample addition.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **15 µL of rehydrated master mix** to the bottom of **26 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. To each well containing 15 µL of rehydrated master mix, add **5 µL of direct patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips and/or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems QuantStudio™ 5 template file for Direct Patient Sample addition (500003XMPDirectQS5v2.edt), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Applied Biosystems QuantStudio™ 5** for download instructions. To manually program the thermocycler, please refer to **Appendix A**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE

Applied Biosystems 7500 Fast Dx



1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 26 samples to be tested by direct sample addition.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **15 µL of rehydrated master mix** to the bottom of **26 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. To each well containing 15 µL of rehydrated master mix, add **5 µL of direct patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips and/or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems 7500 Fast Dx template file for Direct Patient Sample addition (500003XMPDirect7500Dx.sdt), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Applied Biosystems 7500 Fast Dx** for download instructions. To manually program the thermocycler, please refer to **Appendix B**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE

Bio-Rad CFX96 Touch™

1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 26 samples to be tested by direct sample addition.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **15 µL of rehydrated master mix** to the bottom of **26 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. To each well containing 15 µL of rehydrated master mix, add **5 µL of direct patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:



- a. Open the “Data Analysis” window for your specific run
- b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure **“FAM”** is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter **“500”**
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
- c. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure **“CalRed 610”** is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter **“500”**
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
- d. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure **“Quasar 670”** is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)

- i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
10. Refer to **Table 2** for result interpretation.

Note: For Bio-Rad CFX96 Touch™ protocol and plate files for Direct Patient Sample addition (500003XMPDirectCFX96v2.prcl and 500003XMPDirect_plateCFX96v2.pltd), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Bio-Rad CFX96 Touch™** for download instructions. To manually program the thermocycler, please refer to **Appendix C**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE

Bio-Rad CFX384 Touch™

1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 384-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 26 samples to be tested by direct sample addition.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **15 µL of rehydrated master mix** to the bottom of **26 empty wells** (384-well PCR plate).
6. To each well containing 15 µL of rehydrated master mix, add **5 µL of direct patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. When CFX384 Touch™ run is completed, threshold setting update will need to be completed as follows:
 - a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”





- ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - d. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**250**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
 - e. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
- 10. Refer to **Table 2** for result interpretation.

Note: For Bio-Rad CFX384 Touch™ protocol and plate files for Direct Patient Sample addition (500003XMPDirectCFX384.prcl and 500003XMPDirect_plateCFX384.pltd), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Bio-Rad CFX384 Touch™** for download instructions. To manually program the thermocycler, please refer to **Appendix D**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE

Follow the “RT-PCR Set-up - Extracted Patient Sample” section below for the appropriate real-time PCR platform (i.e. QuantStudio™ 5, 7500 Fast Dx, CFX96 Touch™ and CFX384 Touch™). For the validated QIAGEN QIAamp Viral RNA Mini Kit extraction method (manual), pipette 140µL of specimen into the extraction tube/plate and proceed with the protocol as per the manufacturer’s instructions for use (Purification of Viral RNA (Spin Protocol)). Elute each sample in 50µL RNase-free water. After extraction and elution, add **5 µL** of the eluate to the RT-PCR master mix.

Applied Biosystems QuantStudio™ 5



1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 40 samples to be tested using purified nucleic acid samples.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **10 µL of rehydrated master mix** to the bottom of **40 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. To each well containing 10 µL of rehydrated master mix, add **5 µL of extracted patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips and/or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems QuantStudio™ 5 template file for Extracted Patient Sample addition (500003XMPExtractQS5v2.edt), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Applied Biosystems QuantStudio™ 5** for download instructions. To manually program the thermocycler, please refer to **Appendix A**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE

Applied Biosystems 7500 Fast Dx

1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 40 samples to be tested using purified nucleic acid samples.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **10 µL of rehydrated master mix** to the bottom of **40 empty wells** (8-tube PCR strips or 96-well PCR plate).

6. To each well containing 10 μ L of rehydrated master mix, add **5 μ L of extracted patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips and/or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems 7500 Fast Dx template file for Extracted Patient Sample addition (500003XMPExtract7500Dx.sdt), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Applied Biosystems 7500 Fast Dx** for download instructions. To manually program the thermocycler, please refer to **Appendix B**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE

Bio-Rad CFX96 Touch™



1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 8-tube PCR strips or 96-well PCR plates.
3. Using 1000 μ L pipet tip, transfer **400 μ L of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 40 samples to be tested using purified nucleic acid samples.
4. Mix by gently pipetting up and down with 1000 μ L pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **10 μ L of rehydrated master mix** to the bottom of **40 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. To each well containing 10 μ L of rehydrated master mix, add **5 μ L of extracted patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:
 - a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold

- adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “500”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
- c. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “500”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
- d. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “500”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment

10. Refer to **Table 2** for result interpretation.

Note: For Bio-Rad CFX96 Touch™ protocol and plate files for Extracted Patient Sample addition (500003XMPExtractCFX96v2.prcl and 500003XMPExtract_plateCFX96v2.pltd), please refer to the section: “**Instrument Templates and Programming**”: **Install the BioGX Protocol on the Bio-Rad CFX96 Touch™** for download instructions. To manually program the thermocycler, please refer to **Appendix C**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE

Bio-Rad CFX384 Touch™



1. Wear nitrile gloves when handling Sample-Ready™ lyophilized reagents to reduce the generation of static charges. DO NOT use latex gloves.
2. Prepare the appropriate number of 384-well PCR plates.

3. Using 1000 µL pipet tip, transfer **400 µL of molecular grade water** to one vial of lyophilized BioGX Xfree™ COVID-19 reagents. The rehydrated master mix is more than sufficient for 40 samples to be tested using purified nucleic acid samples.
4. Mix by gently pipetting up and down with 1000 µL pipet tip. (**IMPORTANT:** Keep rehydrated master mix in a cold block or on ice if set-up cannot be completed within 20 minutes. **If the rehydrated master mix cannot be used immediately, it should be discarded.**)
5. Transfer **10 µL of rehydrated master mix** to the bottom of **40 empty wells** (384-well PCR plate).
6. To each well containing 10 µL of rehydrated master mix, add **5 µL of direct patient sample**.
7. Affix the appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate or tube to mix and bring liquid to the bottom.
9. When CFX384 Touch™ run is completed, threshold setting update will need to be completed as follows:
 - a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - c. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**250**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
 - d. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
10. Refer to **Table 2** for result interpretation.

Note: For Bio-Rad CFX384 Touch™ protocol and plate files for Extracted Patient Sample addition (500003XMPExtractCFX384.prcl and 500003XMPExtract_plateCFX384.pltd), please refer to the section: **“Instrument Templates and Programming”**: **Install the BioGX Protocol on the Bio-Rad CFX96 Touch™** for download instructions. To manually program the thermocycler, please refer to **Appendix D**.

QUALITY CONTROL

Controls to Monitor Assay Performance

CONTROL

- Each BioGX Xfree™ COVID-19 kit includes molecular primers and probes specific for the detection of the human RNase P gene, a sample processing control (SPC), in the lyophilized RT-PCR master mix. RNase P should be present and detected in each patient sample. In addition, a non-naturally occurring single-stranded RNA is integrated into the lyophilized reagent to serve as a PCR internal amplification control (IAC). The IAC should be present and detected in every control and SARS-CoV-2 negative patient sample. No external addition of the SPC or IAC is required.

External positive and negative controls are available separately for the BioGX Xfree™ COVID-19 kit. The external positive control available from BioGX (BioGX Synthetic RNA Nucleocapsid Phosphoprotein gene region (N1) (BioGX catalog no. 720-0206) is treated as if it were a patient sample and should be positive for both the COVID-19 N1 target and the IAC target. As an alternative, inactivated SARS-CoV-2 RNA available from Zeptometrix (catalog number NATSARS(CoV2)-ST) may also be used as an external positive control and should also be positive for both the COVID-19 N1 target and the IAC target. It is recommended that one external positive control be included with each RT-PCR run of patient samples. The external negative control should consist of molecular grade water and should be positive only for the IAC target. It is recommended that one external negative control be included in each extraction batch (as applicable) and each RT-PCR run of patient samples. BioGX recommends that the external negative control be prepared prior to the external positive control in order to reduce the potential for contamination as a result of control preparation.

Preparation of BioGX External Control for Direct or Extracted Sample Testing

Positive Control [BioGX SARS-CoV-2 (N1)]

Preparation of BioGX SARS-CoV-2 Synthetic Nucleocapsid Phosphoprotein Gene Region (N1) external control (catalog no. 720-0206):

A single BioGX lyophilized SARS-CoV-2 (N1) control bead containing 100,000 copies/bead should be serially diluted as described in the procedure below to achieve a final concentration of 10,000 copies/mL (10 copies/ μ L).

1. Rehydrate one BioGX lyophilized SARS-CoV-2 (N1) control bead with 100 μ L of molecular grade water. Slowly pipette up and down 5 times to homogenize and discard pipette tip. This will be the SARS-CoV-2 (N1) stock solution.
2. With a fresh pipette tip, prepare two microcentrifuge tubes for serial dilution by adding 90 μ L of molecular grade water to each. Label the tube caps with “N1-10” and “N1-100” to represent the 1/10 dilution and 1/100 dilution of SARS-CoV-2 (N1) stock solution, respectively.
3. With a fresh pipette tip, transfer 10 μ L of the SARS-CoV-2 (N1) stock solution to the first microcentrifuge tube labeled “N1-10”. Slowly pipette up and down 5 times to homogenize then discard the pipette tip.
4. With a fresh pipette tip, transfer 10 μ L of the “N1-10” dilution to the second microcentrifuge tube labeled “N1-100”. Slowly pipette up and down 5 times to homogenize then discard the pipette tip. The microcentrifuge tube labeled “N1-100” now contains SARS-CoV-2 (N1) control at 10,000 copies/mL.

Add 5 μ L of SARS-CoV-2 (N1) control (50 copies/PCR reaction) from the microcentrifuge tube labeled “N1-100” to each positive control well position(s) containing dispensed master mix.

Negative Control (No Template) - Add 5 μ L of molecular grade water to (1) well position containing dispensed master mix.

Preparation of Zeptomatrix External Positive Control for Extracted or Direct Samples

Preparation of ZeptoMetrix SARS-Related Coronavirus 2 (SARS-CoV-2 Isolate: USA-WA1/2020)
Zeptomatrix catalog number: NATSARS(COV2)-ST

ZeptoMetrix SARS-Related Coronavirus 2 (SARS-CoV-2) stock concentration of 1,000,000 (1,000 copies/ μ L) should be serially diluted to achieve a final concentration of 10,000 copies/mL (10 copies/ μ L) for the extracted method

1. With a fresh pipette tip, prepare **two** microcentrifuge tubes for serial dilution by adding 18 μ L of molecular grade water to each tube. Label the tube caps with “N1-10” and “N1-100” to represent the 10 times dilution and 100 times dilution of stock to yield 100 copies / μ L and 10 copies / μ L, respectively.
2. With a fresh pipette tip, transfer 2 μ L of the ZeptoMetrix SARS-Related Coronavirus 2 (SARS-CoV-2) stock solution to the first microcentrifuge tube labeled “ZM-10”. Slowly pipette up and down 5 times to homogenize then discard the pipette tip.
3. With a fresh pipette tip, transfer 2 μ L of the “N1-10” dilution to the second microcentrifuge tube labeled “N1-100”. Slowly pipette up and down 5 times to homogenize then discard the pipette tip. The microcentrifuge tube

labeled “N1-100” now contains ZeptoMetrix SARS-Related Coronavirus 2 (SARS-CoV-2) control at 10,000 copies/mL.

4. Add 5 µL of SARS-CoV-2 (N1) control (50 copies/PCR reaction) from the microcentrifuge tube labeled “N1-100” to each positive control well position(s) containing dispensed master mix.

Note: The BioGX SARS-CoV-2 and Zeptomatrix SARS-CoV-2 positive controls, when utilized as described (3,333 copies/mL) with the extracted workflow, may not sufficiently evaluate integrity of the assay reagents and could lead to false specimen results.

For further reference, please reference the product insert for **Lyophilized Control Template Beads (BioGX Product Number Series 720-0206)** which is available for download at www.biogx.com by using the drop down menu at the top right of the home page. Select "Education Center" then select "Product Documents". Choose the appropriate product number under "Template Controls".

RESULTS INTERPRETATION

Expected Results for Kit Controls

All test controls should be examined prior to interpretation of patient results. If the controls do not yield the expected results as shown in **Table 1** below, the patient results cannot be interpreted.

Table 1. Interpretation of BioGX external controls.

Control Type	Applicability for Monitoring	N1 Gene	N1 Expected Ct	RNase P	RNase P Expected Ct	IAC	IAC Expected Ct
Negative Control - Addition of 5 µL Water	Reagent and/or environmental contamination	-	Not Detected	-	Not Detected	+	Ct≤30
N1 Positive Control - BioGX Synthetic Template Control	Substantial reagent failure including primer and probe integrity	+	Ct≤40	-	Not Detected	+	Ct≤30

An external negative control that yields a positive result for the COVID-19 N1 gene or RNase P gene is indicative of errant sample handling and/or contamination. If the N1

target or RNase P target is amplified, the assay should be repeated with reagents from an unopened pouch or new assay kit.

An external positive control that yields a negative result for the COVID-19 N1 target is indicative of a specimen handling and preparation and/or reagent problem. If N1 is not amplified, first review the specimen handling/preparation technique to ID handling/preparation errors then repeat the assay. If the issue persists, repeat with reagents from an unopened pouch or new assay kit.

The IAC, which is present in the RT-PCR master mix, should be detected and produce a Ct value ≤ 30 for all external negative and positive control reactions. If the IAC target is not detected, integrity of the assay components may be compromised, and the assay should be repeated with reagents from an unopened pouch or new assay kit.

Examination and Interpretation of Patient Specimen Results

Assessment of clinical specimen test results should be performed after the external positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted. The list of expected results is outlined in **Table 2**. If results are obtained that do not follow these guidelines, re-test sample or re-extract and re-test the sample if using extraction. If repeat testing yields similar results, collect a fresh sample from the patient for testing. In the presence of a high concentration positive result for the target, the IAC may or may not amplify. This is normal. A positive test result does not necessarily indicate the presence of viable infectious organisms. A positive result is indicative of the presence of target nucleic acid. A negative test result does not preclude the presence of infectious organisms and should not be used as the sole basis for treatment or other patient management decisions.

Table 2. Interpretation of patient sample results¹⁴.

Xfree™ COVID-19 Direct RT-PCR Results	N1 gene		RNase P		IAC		Result Interpretation
	Detect (+/-)	Expected Ct	Detect (+/-)	Expected Ct	Detect (+/-)	Expected Ct	
SARS-CoV-2 POSITIVE	+	Ct \leq 40	+	Ct \leq 36	+/-	Ct \leq 30	Report as POSITIVE
SARS-CoV-2 NEGATIVE	-	Not Detected	+	Ct \leq 36	+	Ct \leq 30	Report as NEGATIVE
Indeterminant	-/+	Ct 0-40	- / Ct \geq 36	-/Ct \geq 36	+	Ct \leq 30	Repeat Test*
Indeterminant	-/+	Ct 0-40	+	Ct \leq 36	- / Ct \geq 30	- / Ct \geq 30	Repeat Test*

*Repeat the test by preparing a new test from the remaining patient sample collection and ensure the patient sample and PCR master mix is dispensed properly in the well being analyzed. Repeat testing must yield RNaseP and IAC detection without N1 detection to confirm a NEGATIVE result. However, if the same result is obtained upon a repeat test, report results as INCONCLUSIVE and request a new sample collection.

REPEAT TESTING FOR INSTRUMENT FAILURE

In case of instrument failure, repeat testing can be performed by setting up a new run using the original sample/specimen as described above in the “Specimen Preparation” section. Refer to the “Troubleshooting” section of the Applied Biosystems QuantStudio™ 5⁹, Applied Biosystems 7500 Fast Dx¹⁰, Bio Rad CFX96 Touch™/CFX384 Touch™ ¹¹ Instruction Manuals for interpretation of warning and error codes.

LIMITATIONS OF THE PROCEDURE

- The use of this assay as an *in vitro* diagnostic under FDA Emergency Use Authorization (EUA) is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.
- This product is intended for use with specimens collected using specimen collection and transport devices listed in the “Equipment and Materials Required But Not Provided” section.
- Incorrect test results may occur from improper specimen collection, handling or storage, technical error, sample mix-up, or because the number of organisms in the specimen is below the analytical sensitivity of the test. Careful compliance with the BioGX Xfree™ COVID-19 assay instructions for use and the real-time PCR platforms (ie. Applied Biosystems QuantStudio™ 5⁹, Applied Biosystems 7500 Fast Dx¹⁰, Bio-Rad CFX96 Touch™/CFX384 Touch™ ¹¹) are necessary to avoid erroneous results.
- Good laboratory technique is essential for the proper performance of this assay.
- The BioGX SARS-CoV-2 positive control, when utilized at 3,333 copies/mL with the extracted workflow, may not sufficiently evaluate integrity of the assay reagents and could lead to false specimen results.
- A positive test result does not necessarily indicate the presence of viable infectious organisms. A positive result is indicative of the presence of target nucleic acid. A negative test result does not preclude the presence of infectious organisms and should not be used as the sole basis for treatment or other patient management decisions.
- The BioGX Xfree™ COVID-19 assay results may sometimes be Unresolved or Indeterminate due to a lack of RNase P and/or IAC detection. Instrument failure could also contribute to an incomplete run result and require retesting that can lead to a delay obtaining final results.

- The performance of the BioGX Xfree™ COVID-19 assay was established using nasopharyngeal swab samples. Anterior nasal, mid-turbinate, and oropharyngeal swab specimens, and nasopharyngeal wash/aspirate or nasal aspirates are also considered acceptable specimen types for use with the BioGX Xfree™ COVID-19 assay but performance has not been established.
- Mutations or polymorphisms in primer- or probe-binding regions may affect detection of new or unknown SARS-CoV-2 variants resulting in a false negative result with the BioGX Xfree™ COVID-19 assay.
- 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.
- The BioGX Xfree™ COVID-19 assay requires the use of three (3) optical channels on the Applied Biosystems QuantStudio™ 5, Applied Biosystems 7500 Fast Dx, Bio-Rad CFX96 Touch™ or Bio-Rad CFX384 Touch™ platforms.

Conditions of Authorization for the Laboratory

The BioGX Xfree™ COVID-19 Direct RT-PCR Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on 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 BioGX Xfree™ COVID-19 Direct RT-PCR assay, the relevant Conditions of Authorization are listed below.

- a) Authorized laboratories¹ using the BioGX Xfree™ COVID-19 Direct RT-PCR assay 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 the BioGX Xfree™ COVID-19 Direct RT-PCR assay must use the BioGX Xfree™ COVID-19 Direct RT-PCR assay as outlined in the authorized labeling. Deviations from the authorized procedures, such as the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use the BioGX Xfree™ COVID-19 Direct RT-PCR assay are not permitted.
- c) Authorized laboratories that receive the BioGX Xfree™ COVID-19 Direct RT-PCR assay must notify the relevant public health authorities of their intent to run the BioGX Xfree™ COVID-19 Direct RT-PCR assay prior to initiating testing.

- d) Authorized laboratories using the BioGX Xfree™ COVID-19 Direct RT-PCR assay must have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- e) Authorized laboratories must collect information on the performance of the BioGX Xfree™ COVID-19 Direct RT-PCR assay and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRHEUA-Reporting@fda.hhs.gov) and BioGX, Inc. (via email: covid19@biogx.com) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the BioGX Xfree™ COVID-19 Direct RT-PCR assay of which they become aware.
- f) All laboratory personnel using the BioGX Xfree™ COVID-19 Direct RT-PCR assay must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit, and use the BioGX Xfree™ COVID-19 Direct RT-PCR assay in accordance with the authorized labeling.
- g) BioGX, Inc. authorized distributors, and authorized laboratories using the BioGX Xfree™ COVID-19 Direct RT-PCR assay must 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.

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.”

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity - Direct Patient Sample

Limit of Detection (LoD) was determined by serially diluting quantified inactivated SARS-CoV-2 (USA-WA1/2020; Zeptomatrix catalog number NATSARS(CoV2)-ST) into negative NP swabs samples in Copan UTM[®]/VTM, Saline, and Copan ESwab[™] transport media. The LoD for contrived samples in saline, Copan ESwab[™], or Copan UTM[®]/VTM was then confirmed by testing additional replicates (n=20) of inactivated SARS-CoV-2 virus. LoD confirmation was performed using the following real-time PCR platforms: Applied Biosystems QuantStudio[™] 5, Applied Biosystems 7500 Fast Dx, Bio-Rad CFX96 Touch[™] and Bio-Rad CFX384 Touch[™]. The LoD for the assay using direct patient samples on the validated platforms ranged from 1,000 - 3,000 copies/mL (**Table 3**).

Table 3. LoD confirmation -Direct Patient Sample

Instrument	Transport Media	Concentration [Copies/ mL]	N1-gene		RNase P	IAC
			Agreement with Expected Result (% Positive)	Mean Ct	Mean Ct	Mean Ct
QuantStudio™ 5	UTM®VTM	1000	19/20 (95%)	35.1	30.0	24.1
	Saline	1000	19/20 (95%)	34.2	31.4	24.4
	ESwab™	3000	20/20 (100%)	34.1	29.2	25.4
7500 Fast Dx	UTM®VTM	1000	20/20 (100%)	34.6	30.6	26.4
	Saline	1000	20/20 (100%)	35.3	30.8	26.2
	ESwab™	2000	20/20 (100%)	34.6	28.8	28.1
CFX96 Touch™	UTM®VTM	1500	19/20 (95%)	36.4	30.4	27.4
	Saline	1500	20/20 (100%)	35.6	33.2	27.9
	ESwab™	1500	19/20 (95%)	35.9	30.1	28.3
CFX384 Touch™	UTM®VTM	1000	19/20 (95%)	36.3	31.2	27.2
	Saline	2000	20/20 (100%)	36.6	32.9	27.5
	ESwab™	3000	20/20 (100%)	36.1	28.6	29.0

Analytical Sensitivity - Extracted Patient Sample

Limit of Detection (LoD) was determined by serially diluting quantified inactivated SARS-CoV-2 (USA-WA1/2020; Zeptomatrix catalog number NATSARS(CoV2)-ST) into negative NP swab samples in saline, Copan ESwab™, or Copan UTM®VTM, which were then extracted with the Qiagen QIAamp Viral RNA Mini Kit as described above. The LoD for contrived samples in saline, Copan ESwab™, or Copan UTM®VTM was then confirmed by testing additional replicates (n=20) of inactivated SARS-CoV-2 virus. LoD confirmation was performed using the following real-time PCR platforms: Applied Biosystems QuantStudio™ 5, Applied Biosystems 7500 Fast Dx, Bio-Rad CFX96 Touch™ and Bio-Rad CFX384 Touch™. The LoD for the assay using extracted patient samples on the validated platforms ranged from 125 - 500 copies/mL (Table 4).

Table 4. LoD confirmation – Extracted Patient Sample

Instrument	Transport Media	Concentration [Copies/ mL]	N1-gene		RNase P	IAC
			Agreement with Expected Result (% Positive)	Mean Ct	Mean Ct	Mean Ct
QuantStudio™ 5	UTM [®] VTM	250	20/20 (100%)	33.9	25.5	23.5
	Saline	250	20/20 (100%)	34.6	29.3	23.5
	ESwab™	500	19/20 (95%)	35.2	28.2	23.5
7500 Fast Dx	UTM [®] VTM	125	19/20 (95%)	35.2	28.4	26.1
	Saline	250	19/20 (95%)	34.7	29.2	26.1
	ESwab™	250	19/20 (95%)	35.3	27.1	26.0
CFX96 Touch™	UTM [®] VTM	250	20/20 (100%)	35.8	25.7	26.7
	Saline	250	20/20 (100%)	36.0	29.5	26.9
	ESwab™	250	19/20 (95%)	36.3	27.1	26.8
CFX384 Touch™	UTM [®] VTM	500	20/20 (200%)	36.7	26.9	26.5
	Saline	500	20/20 (100%)	36.5	29.6	26.4
	ESwab™	500	20/20 (100%)	36.9	29.5	26.6

*Qiagen Viral RNA Mini extraction kit used according to manufacturer recommendations (i.e. 140µL sample processed and eluted in 50µL).

Inclusivity (*in-silico*)

The Xfree™ COVID-19 assay primer and probe sequences are identical to the FDA EUA authorized CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. CDC conducted the *in silico* inclusivity analysis. The data from this analysis is available in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (CDC) Instructions For Use document, to which the CDC has granted a broad right of reference.

An additional *in silico* analysis assessing the impact of the recently emerged SARS-CoV-2 variants on target detection by BioGX showed that none of the observed mutations would be expected to interfere with N gene detection.

Cross-Reactivity (Analytical Specificity)

The Xfree™ COVID-19 assay primer and probe sequences are identical to the FDA EUA authorized CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. CDC conducted the *in silico* cross-reactivity analysis. The data from this analysis is available in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (CDC) Instructions For Use document, to which the CDC has granted a broad right of reference.

Interfering Substances

An interfering substances study was conducted to evaluate the potential of endogenous substances to cause both false positive and false negative results with the Xfree™ COVID-19 assay when using the direct workflow.

For this study, potential interfering substances were spiked into pooled Copan UTM® negative nasopharyngeal swab samples containing no viral RNA or SARS-CoV-2 (USA-WA1/2020; Zeptomatrix catalog number NATSARS(CoV2)-ST) at approximately 5X LoD (5,000 copies/mL). **Table 6** summarizes the results obtained from negative (SARS-CoV-2 Negative NP) and positive (SARS-CoV-2 Positive NP) replicate runs (n=3) that were conducted for each substance. All runs were conducted following the direct sample workflow and analyzed with the both the Applied Biosystems QuantStudio™ 5 and Bio-Rad CFX96 Touch™. All samples yielded the expected results, indicating an absence of interference with the tested substances.

Table 6. Analysis of interfering substances.

Potential Interfering substance	Final concentration (in sample)	Agreement with expected results			
		QuantStudio™ 5		CFX96 Touch™	
		SARS-CoV-2 Positive NP	SARS-CoV-2 Negative NP	SARS-CoV-2 Positive NP	SARS-CoV-2 Negative NP
None	N/A	100%	100%	100%	100%
Mucin (bovine submaxillary gland, type I-S)	2.5 mg/mL	100%	100%	100%	100%
Mucin (bovine submaxillary gland, type I-S)	1.25 mg/mL	100%	100%	100%	100%
Nasacort	10% v/v	100%	100%	100%	100%
NeilMed NasoGEL	1.25% w/v	100%	100%	100%	100%

Similasan Nasal Allergy Relief™	15% v/v	100%	100%	100%	100%
Saline Nasal Spray	15% v/v	100%	100%	100%	100%
Act dry mouth lozenges	0.04 g/mL	100%	100%	100%	100%
Chloraseptic® Spray	10% v/v	100%	100%	100%	100%
Cepacol Extra Strength Cherry Lozenges	0.046 g/mL (0.15mg/mL Benzocaine)	100%	100%	100%	100%
Chloraseptic Lozenges	0.071 g/mL (0.12mg/mL Benzocaine)	100%	100%	100%	100%
Halls Cough drops	0.062 g/mL (0.15 mg/mL Menthol)	100%	100%	100%	100%
Nature's Bounty® Zinc (50mg)	10µg/mL	100%	100%	100%	100%
Nyquil	10% v/v	100%	100%	100%	100%
Robitussin	10% v/v	100%	100%	100%	100%
Flonase	5% v/v	100%	100%	100%	100%
Ethanol	5% v/v	100%	100%	100%	100%
ORIGINAL LISTERINE® Mouthwash	5% v/v	100%	100%	100%	100%
Colgate Toothpaste Original	1% v/v	100%	100%	100%	100%
Mupirocin Ointment (Bactroban)	5 mg/mL	100%	100%	100%	100%
Tobramycin (Antibiotic)	100 ng/mL	100%	100%	100%	100%
Oseltamivir (Tamiflu API)	3.3 mg/mL	100%	100%	100%	100%

Emergen-C Vitamin C Drink - Super Orange	10% v/v	100%	100%	100%	100%
Petroleum jelly (Vaseline)	1% w/v	100%	100%	100%	100%
Tobacco	1% v/v	100%	100%	100%	100%

Clinical Performance

Positive percent agreement (PPA) and negative percent agreement (NPA) between the BioGX Xfree™ COVID-19 assay and a highly sensitive FDA EUA authorized RT-PCR comparator was determined using 60 individual SARS-CoV-2 positive and 35 individual SARS-CoV-2 negative clinical nasopharyngeal swab samples collected in UTM/VTM and collected by qualified healthcare professionals from individuals suspected of SARS-CoV-2 infection. Samples were handled as described in the package insert of the collection device and stored frozen until use.

Testing with Direct Patient Sample Addition

The clinical samples were tested by direct sample addition (5 µL of clinical sample transferred directly into 15 µL of the BioGX Xfree™ COVID-19 mastermix). Clinical samples were analyzed on the QuantStudio™ 5 following the device instructions for use. The BioGX Xfree™ COVID-19 assay, when using the direct sample addition workflow achieved a PPA of 95% and an NPA of 100% when compared to the comparator method (**Table 7**).

Testing with Extracted Patient Samples

The clinical samples were also tested following the extracted patient sample workflow with the Qiagen QIAamp Viral RNA Mini Kit following the manufacturer's recommendations, where 140 µL of clinical sample were extracted and then eluted with 50 µL RNase-free water. The eluted nucleic acid template (5 µL) was added to 10 µL of the BioGX Xfree™ master mix. Positive clinical patient samples (n=60) and negative clinical patient samples (n=35) yielded a PPA and NPA of 100% and 94.29%, respectively, when analyzed on the QuantStudio™ 5 (**Table 8**).

Table 7. Clinical performance of the BioGX Xfree™ COVID-19 direct sample addition workflow

BioGX Xfree™ COVID-19 Assay (Direct Sample Addition)	Comparator RT-PCR EUA Test	
	Positive	Negative
Positive	57	0
Negative	3*	35
Positive Percent Agreement (95%CI)	95.0% (86.3-98.3%)	
Negative Percent Agreement (95%CI)	100.0% (90.1-100.0%)	

*The three direct samples with discordant results were identified as positive by the comparator method with Ct values between 37.6 & 40.5.

Table 8. Clinical performance of the BioGX Xfree™ COVID-19 extracted sample workflow

BioGX Xfree™ COVID-19 Assay (Extracted Sample Workflow)	Comparator RT-PCR EUA Test	
	Positive	Negative
Positive	60	2*
Negative	0	33
Positive Percent Agreement	100.0% (94.0-100.0%)	
Negative Percent Agreement	94.3% (81.4-98.4%)	

*The two samples that tested positive with the BioGX Xfree™ COVID-19 assay and negative with the comparator method was tested with a third, highly sensitive RT-PCR EUA authorized test. Both samples tested positive with the second RT-PCR EUA authorized test.

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APPENDIX A: Applied Biosystems QuantStudio™ 5 Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5** manual programming specific for Direct Patient sample testing.

1. Open the QuantStudio Design and Analysis Software v1.5.1
2. Select the “Create New Experiment” option
3. Within the properties tab, select the following properties to filter:
 - a. Name: User defined or 500003XMPDirectQS5v2
 - b. Instrument Type: QuantStudio 5 System
 - c. Block Type: 96-Well 0.2 mL
 - d. Experiment type: Standard Curve
 - e. Chemistry: TaqMan Reagents
 - f. Run mode: Standard
4. Run Method (Method Tab)
 - a. Change the Reaction Volume to **20.0 µL**
 - b. Temperature of the enabled heated cover will remain at 105.0 °C
 - c. Within the “PCR Stage “ section of the thermocycling parameters, an additional step will need to be added by bringing the mouse pointer above “Step 2” will allow the “+” and “-” buttons to become visible.
 - d. Click the “+” button to add “Step 3” to the “PCR Stage”.
 - e. Going back to the first hold stage enter the following parameters (**Table A1**):
 - i. Hold Stage: Step 1
 1. 1.6 ramp rate
 2. 46°C
 3. 20 minutes
 - ii. Hold Stage: Step 2
 1. 1.6 ramp rate
 2. 95°C
 3. 5 minutes
 - iii. PCR Stage: Step 1
 1. 1.6 ramp rate
 2. 95°C
 3. 3 seconds

- iv. PCR Stage: Step 2
 - 1. 1.6 ramp rate
 - 2. 62°C
 - 3. 20 seconds
 - 4. Scanning is turned ON by clicking the camera icon (dark blue when ON).
- v. PCR Stage: Step 3
 - 1. 1.6 ramp rate
 - 2. 70°C
 - 3. 5 seconds
 - 4. Scanning is turned OFF by clicking the camera icon (light blue when OFF).
- vi. Number of cycles set to 40 (located at the bottom of the “PCR Stage” section).

Table A1. QuantStudio™ 5 thermocycling summary - Direct Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Ramp Rate (°C/s)	1.6	1.6	1.6	1.6	1.6
Temperature (°C/s)	46	95	95	62	70
Duration (Min:sec)	20:00	5:00	0:03	0:20	0:05
Detection	OFF	OFF	OFF	ON	OFF
No. of PCR Cycles	HOLD	HOLD	40		

5. Plate Setup (Plate Tab)

- a. Under the “Plate Attribute” section of the Quick Setup tab, change the Passive Reference to “NONE”.
- b. On the left side of the screen (next to Quick Setup), choose “Advanced Setup”.
- c. In the “Targets” section, click on the “Add” box and add two targets for a total of three. Rename Target1 as “RNaseP”.
- d. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “FAM”.

- e. Click on the “Target 2” box and change the name to “N1”.
 - f. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “ROX”.
 - g. Click on the “Target 3” box and change the name to “IAC”.
 - h. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “Cy5”
 - i. Ensure that the “Quencher” tab for all three targets is set to “None”.
 - j. Highlight all the wells that you intend to use and ensure that the boxes besides the three targets are selected and in the task select “U” for unknown. This should populate the plate on the right side of the screen with each target.
 - k. To name your wells by sample name, select the appropriate well on the right side of the screen. Under the “Samples” tab on the left side, click on the current sample name and rename to whatever is desired for the experiment. Check the box besides the sample name: the names will populate on the right side.
 - l. To add additional sample names, click “Add” on the samples tab and repeat the instructions above.
 - m. Click on the “Results” tab on the top of the screen
6. Threshold Setup (Results Tab)
- a. Navigate to the “Results” tab and select the settings wheel besides “Analyze” in the upper right corner.
 - b. Click on target “N1” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 10,000.**
 - c. Click on target “RNaseP” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 25,000.**
 - d. Click on target “IAC” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 5,000.**
 - e. Click “Apply” in the bottom right corner.
7. Save File
- a. Navigate to the “File” tab and choose “Save As”. Name the file ‘500003XMPDirectQS5v2’ and ensure it is saved as an *.edt file.
8. Run File
- a. Select “File” then “Open”.
 - b. Select your chosen *.edt file.
 - c. Navigate to the “Run” tab, and click the dropdown arrow next to “Start Run”. Ensure the name of the instrument is your preferred instrument. You will be prompted to enter a name for the run.
 - d. Insert the desired run name and click “Start Run”.

Applied Biosystems QuantStudio™ 5 - Thermocycling Programming

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5** manual programming specific for Extracted Patient sample testing.

1. Open the QuantStudio Design and Analysis Software v1.5.1
2. Select the “Create New Experiment” option
3. Within the properties tab, select the following properties to filter:
 - a. Name: User defined or 500003XMPExtractQS5v2
 - b. Instrument Type: QuantStudio 5 System
 - c. Block Type: 96-Well 0.2 mL
 - d. Experiment type: Standard Curve
 - e. Chemistry: TaqMan Reagents
 - f. Run mode: Standard
4. Run Method (Method Tab)
 - a. Change the Reaction Volume to **15.0 µL**
 - b. Temperature of the enabled heated cover will remain at 105.0 °C
 - c. Within the “PCR Stage “ section of the thermocycling parameters, an additional step will need to be added by bringing the mouse pointer above “Step 2” will allow the “+” and “-” buttons to become visible.
 - d. Click the “+” button to add “Step 3” to the “PCR Stage”.
 - e. Going back to the first hold stage enter the following parameters (**Table A1**):
 - i. Hold Stage: Step 1
 1. 1.6 ramp rate
 2. 46°C
 3. 20 minutes
 - ii. Hold Stage: Step 2
 1. 1.6 ramp rate
 2. 95°C
 3. 5 minutes
 - iii. PCR Stage: Step 1
 1. 1.6 ramp rate
 2. 95°C
 3. 3 seconds
 - iv. PCR Stage: Step 2
 1. 1.6 ramp rate
 2. 62°C
 3. 20 seconds
 4. Scanning is turned ON by clicking the camera icon (dark blue when ON).
 - v. PCR Stage: Step 3

1. 1.6 ramp rate
 2. 70°C
 3. 5 seconds
 4. Scanning is turned OFF by clicking the camera icon (light blue when OFF).
- vi. Ensure the number of cycles is set to 40 (located at the bottom border of the PCR Stage).

5. Plate Setup (Plate Tab)

- a. Under the “Plate Attribute” section of the Quick Setup tab, change the Passive Reference to “NONE”.
- b. On the left side of the screen (next to Quick Setup), choose “Advanced Setup”.
- c. In the “Targets” section, click on the “Add” box and add two targets for a total of three. Rename Target1 as “RNaseP”.
- d. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “FAM”.
- e. Click on the “Target 2” box and change the name to “N1”.
- f. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “ROX”.
- g. Click on the “Target 3” box and change the name to “IAC”.
- h. Click the associated reporter box below the Reporter tab and, from the drop down menu, choose “Cy5”
- i. Ensure that the “Quencher” tab for all three targets is set to “None”.
- j. Highlight all the wells that you intend to use and ensure that the boxes besides the three targets are selected. In the “Task” section select “U” for unknown. This should populate the plate on the right side of the screen with each target.
- k. To name your wells by sample name, select the appropriate well on the right side of the screen. Under the “Samples” tab on the left side, click on the current sample name and rename to whatever is desired for the experiment. Check the box besides the sample name: the names will populate on the right side.
- l. To add additional sample names, click “Add” on the samples tab and repeat the instructions above.
- m. Click on the “Results” tab on the top of the screen

6. Threshold Setup (Results Tab)

- a. Navigate to the “Results” tab and select the settings wheel besides “Analyze” in the upper right corner.
- b. Click on target “N1” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 10,000.**
- c. Click on target “RNaseP” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 25,000.**
- d. Click on target “IAC” and uncheck the “Ct Settings to Use: Default Settings” box directly to the right. Then uncheck the “Automatic Threshold” box: **set the threshold to 5,000.**
- e. Click “Apply” in the bottom right corner.

7. Save File

- a. Navigate to the “File” tab and choose “Save As”. Name the file ‘500003XMPExtractQS5v2’ and ensure it is saved as an *.edt file.

8. Run File

- a. Select “File” then “Open”.
- b. Select your chosen *.edt file.
- c. Navigate to the “Run” tab, and click the dropdown arrow next to “Start Run”. Ensure the name of the instrument is your preferred instrument. You will be prompted to enter a name for the run.
- d. Insert the desired run name and click “Start Run”.

APPENDIX B: Applied Biosystems 7500 Fast Dx - Thermocycling Programming

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for Applied Biosystems 7500 Fast Dx manual programming specific for Direct Patient sample testing.

1. Open the Applied Biosystems 7500 Fast Dx software v1.4.1
2. Go to the “Tools” tab and navigate to “Detector Manager”
 - a. Click on the “File” dropdown and select “New”.
 - b. Name the target as “N1”
 - i. Set the reporter dye to “ROX”
 - ii. Set the quencher dye to none
 - iii. Click Apply
 - c. Name the target as “RNaseP”
 - i. Set the reporter dye to “FAM”
 - ii. Set the quencher dye to “None”
 - iii. Click Apply
 - d. Name the target as “IAC”
 - i. Set the reporter dye to “Cy5”
 - ii. Set the quencher dye to “None”
 - iii. Click Apply
3. Click the “File” tab and select “New”
4. On the “Define document” Pop-Up:
 - a. Assay: Standard Curve (Absolute Quantification)
 - b. Container: 96 Well Clear
 - c. Template: Blank Document
 - d. Run Mode: Fast 7500
 - e. Operator: Select the operator you wish to run the assay with
 - f. Plate Name: Select your desired name for the experiment file
 - g. Click “Next”
5. Select the previously created detectors from the library: N1, RNaseP, IAC and click add.
6. Set the Passive Reference to “None”
7. Click “next”. Select all the wells and assign the targets to them.
8. Select Finish.
9. Navigate to the Instrument tab.
 - a. Change the Sample Volume to **20.0 µL**
 - b. Click the “Add Hold” button until you have two hold steps. Set the number of repetitions to 1.
 - c. Click the “Add Step” button until you have three PCR cycling steps. Set the number of repetitions for this stage to 40.
 - d. Going back to the first hold stage enter, the following parameters (**Table B1**):
 - i. Hold Stage: Step 1

1. Auto ramp rate
 2. 46°C
 3. 20 minutes
 - ii. Hold Stage: Step 2
 1. Auto ramp rate
 2. 95°C
 3. 5 minutes
 - iii. PCR Stage: Step 1
 1. Auto ramp rate
 2. 95°C
 3. 3 seconds
 - iv. PCR Stage: Step 2
 1. Auto ramp rate
 2. 62°C
 3. 24 seconds.
 - v. PCR Stage: Step 3
 1. Auto ramp rate
 2. 70°C
 3. 5 seconds
 - vi. Under settings, select the following:
 1. Sample Volume: 20
 2. Run Mode: Fast 7500
 3. Data Calculation: Stage 3, step 2
- e. Select the “Analysis” tab and click “Analysis settings”.
 - i. Select N1’s target settings by clicking on the detector dropdown.
 1. Select “Manual CT” and **set the threshold to 50,000**
 2. Select “Manual Baseline” and set the start cycle to 3 and the end to 10.
 3. Click apply
 - ii. Select RNaseP
 1. Select Manual CT and **set the threshold to 100,000**
 2. Select “Manual Baseline” and set the start cycle to 3 and the end to 15
 3. Click apply
 - iii. Select IAC
 1. Select Manual CT and **set the threshold to 100,000**
 2. Select “Manual Baseline” and set the start cycle to 3 and the end to 20
 3. Click “Apply”, then select “OK”.
- f. Select File -> Save. Set your desired name and your template is saved.

Table B1. Applied Biosystems 7500 thermocycling summary - Direct Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Ramp Rate (°C/s)	Auto	Auto	Auto	Auto	Auto
Temperature (°C/s)	46	95	95	62	70
Duration (Min:sec)	20:00	5:00	0:03	0:24	0:05
Detection	OFF	OFF	OFF	ON	OFF
No. of PCR Cycles	HOLD	HOLD	40		

Applied Biosystems 7500 Fast Dx- Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **Applied Biosystems 7500 Fast Dx** manual programming specific for Extracted patient sample testing.

1. Open the Applied Biosystems 7500 Fast Dx software v1.4.1
2. Go to the “Tools” tab and navigate to “Detector Manager”
 - a. Click on the “File” dropdown and select “New”.
 - b. Name the target as “N1”
 - i. Set the reporter dye to “ROX”
 - ii. Set the quencher dye to none
 - iii. Click Apply
 - c. Name the target as “RNaseP”
 - i. Set the reporter dye to “FAM”
 - ii. Set the quencher dye to “None”
 - iii. Click Apply
 - d. Name the target as “IAC”
 - i. Set the reporter dye to “Cy5”
 - ii. Set the quencher dye to “None”
 - iii. Click Apply
3. Click the “File” tab and select “New”
4. On the “Define document” Pop-Up:
 - a. Assay: Standard Curve (Absolute Quantification)
 - b. Container: 96 Well Clear

- c. Template: Blank Document
 - d. Run Mode: Fast 7500
 - e. Operator: Select the operator you wish to run the assay with
 - f. Plate Name: Select your desired name for the experiment file
 - g. Click “Next”
5. Select the previously created detectors from the library: N1, RNaseP, IAC and click add.
 6. Set the Passive Reference to “None”
 7. Click “next”. Select all the wells and assign the targets to them.
 8. Select Finish.
 9. Navigate to the Instrument tab.
 - a. Change the Sample Volume to **15.0 µL**
 - b. Click the “Add Hold” button until you have two hold steps. Set the number of repetitions to 1.
 - c. Click the “Add Step” button until you have three PCR cycling steps. Set the number of repetitions for this stage to 40.
 - d. Going back to the first hold stage enter, the following parameters (**Table B1**):
 - i. Hold Stage: Step 1
 1. Auto ramp rate
 2. 46°C
 3. 20 minutes
 - ii. Hold Stage: Step 2
 1. Auto ramp rate
 2. 95°C
 3. 5 minutes
 - iii. PCR Stage: Step 1
 1. Auto ramp rate
 2. 95°C
 3. 3 seconds
 - iv. PCR Stage: Step 2
 1. Auto ramp rate
 2. 62°C
 3. 24 seconds.
 - v. PCR Stage: Step 3
 1. Auto ramp rate
 2. 70°C
 3. 5 seconds
 - vi. Under settings, select the following:
 1. Sample Volume: 20
 2. Run Mode: Fast 7500
 3. Data Calculation: Stage 3, step 2
 - e. Select the “Analysis” tab and click “Analysis settings”.
 - i. Select N1’s target settings by clicking on the detector dropdown.
 1. Select “Manual CT” and **set the threshold to 50,000**
 2. Select “Manual Baseline” and set the start cycle to 3 and the end to 10.
 3. Click apply
 - ii. Select RNaseP

1. Select Manual CT and **set the threshold to 100,000**
2. Select “Manual Baseline” and set the start cycle to 3 and the end to 15
3. Click apply
- iii. Select IAC
 1. Select Manual CT and **set the threshold to 100,000**
 2. Select “Manual Baseline” and set the start cycle to 3 and the end to 20
 3. Click “Apply”, then select “OK”.
- f. Select File -> Save. Set your desired name and your template is saved.

APPENDIX C: Bio-Rad CFX96 Touch™ - Thermocycling Programming

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for CFX96 Touch™ manual programming specific for Direct Patient sample testing.

1. Launch the CFX Maestro software.
2. Select “File”, then choose “New” and select “Protocol”
3. Program thermocycling conditions in the Protocol Editor
 - a. Set Sample Volume to **20 µL**
 - b. Click “Insert Step” to add a total of five total steps and program as follows (**Table C1**):
 - c. Step 1
 - i. Temp: 46°C
 - ii. Time: 20:00
 - d. Step 2
 - i. Temp: 95°C
 - ii. Time: 5:00
 - e. Step 3
 - i. Temp: 95°C
 - ii. Time: 0:03
 - f. Step 4
 - i. Temp: 62°C
 - ii. Time: 0:20
 - iii. Click “Add Plate Read to Step”. Also, ensure this is the only step containing a plate read
 - g. Step 5
 - i. Temp: 70°C
 - ii. Time: 0:05
 - h. Step 6
 - i. Click to highlight “GOTO” and enter “3” to indicate thermocycling includes Step 3, Step 4 and Step 5.
 - ii. At “GOTO 3”, confirm “39 more times” is programmed for a total of 40 cycles.
 - I. Save the new cycling conditions as protocol for future use
 - i. At the upper left of the screen select the Save button
 - ii. Save in the ExpressLoad folder
 - iii. Name the file ‘500003XMPDirectCFX96v2’
 - iv. Save as type ‘Protocol File (*.prcl)’
 - v. Select Save
 - vi. Click Ok in the protocol editor window

Table C1. CFX96 Touch™ thermocycling summary - Direct Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Temperature (°C/s)	46	95	95	62	70
Duration (Min:sec)	20:00	5:00	0:03	0:20	0:05
Detection	OFF	OFF	OFF	ON	OFF
No. of PCR Cycles	HOLD	HOLD	40		

4. Define the plate setup

- a. Select “File” then choose “New” and select “Plate”
- b. In the Plate Editor, click settings and ensure your settings are as follows
 - i. Plate Size: 96 wells
 - ii. Plate Type: BR White OR BR Clear (BioGX recommends BR White)
- c. Highlight all wells and choose “Select Fluorophores”
 - i. Uncheck all currently selected wells and check ONLY the following: FAM, Cal Red 610, Quasar 670
- d. For “Sample Type” select “Unknown”
- e. Check the boxes besides all three of your selected fluorophores. Change the target name as follows:
 - i. FAM: RNaseP
 - ii. Cal Red 610: N1
 - iii. Quasar 670: IAC
- f. Save the new plate setup for future use
 - i. At the upper left of the screen select “File” then select “Save”
 - iii. Name the file ‘500003XMPDirect_plateCFX96v2’
 - iv. Save as type ‘Plate File (*.pltd)’
 - v. Select Save
 - vi. Click Ok in the Plate Editor window
- g. Saving Protocol
 - i. Select “File” then “Open”
 - ii. Select “Protocol” then open your saved protocol.
 - ii. Ensure the PCR steps are correct and click “OK”
 - iii. Click the “Plate” tab and choose “Select Existing”. Choose the plate that was saved.
 - iv. Add any desired notes and click on the “Start Run” tab.
 - v. Save the run with the desired name
- h. Assigning a sample name to well positions

- Once the run has begun, reaction wells can be assigned with a unique sample name as follows:
- i. Navigate to “Real Time Status”
 - ii. On the top-middle section of the screen, click “Plate Set-Up” followed by “View/Edit Plate”.
 - iii. Click “Spreadsheet View/Importer” on the upper right quadrant of the screen. Wells can be named with unique identifiers for Sample Name, Sample Type, etc.
5. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:
- a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - c. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
 - d. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
6. Refer to **Table 2** for result interpretation.

Bio-Rad CFX96 Touch™ - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **CFX96 Touch™** manual programming specific for Extracted Patient sample testing.

1. Launch the CFX Maestro software.
2. Choose “File”, then choose “New” and select “Protocol”
3. Program thermocycling conditions in the Protocol Editor
 - a. Set Sample Volume to **15 µL**
 - b. Click “Insert Step” to add a total of five total steps and program as follows (**Table C1**):
 - c. Step 1
 - i. Temp: 46°C
 - ii. Time: 20:00
 - d. Step 2
 - i. Temp: 95°C
 - ii. Time: 5:00
 - e. Step 3
 - i. Temp: 95°C
 - ii. Time: 0:03
 - f. Step 4
 - i. Temp: 62°C
 - ii. Time: 0:20
 - iii. Click “Add Plate Read to Step”. Also, ensure this is the only step containing a plate read
 - g. Step 5
 - i. Temp: 70°C
 - ii. Time: 0:05
 - h. Step 6
 - i. Click to highlight “GOTO” and enter “3” to indicate thermocycling includes Step 3, Step 4 and Step 5.
 - ii. At “GOTO3”, confirm “39 more times” is programmed for a total of 40 cycles.
 - i. Save the new cycling conditions as a protocol for future use
 - i. At the upper left of the screen select the Save button
 - ii. Save in the ExpressLoad folder
 - iii. Name the file ‘500003XMPExtractCFX96v2’
 - iv. Save as type ‘Protocol File (*.prcl)’
 - v. Select Save
 - vi. Click Ok in the protocol editor window
4. Define the plate setup
 - a. Select “File” then choose “New” and select “Plate”
 - b. In the Plate Editor, click settings and ensure your settings are as follows
 - i. Plate Size: 96 wells
 - ii. Plate Type: BR White OR BR Clear (BioGX recommends BR White)

- c. Highlight all wells and choose “Select Fluorophores”
 - i. Uncheck all currently selected wells and check ONLY the following: FAM, Cal Red 610, Quasar 670
 - d. For “Sample Type” select “Unknown”
 - e. Check the boxes besides all three of your selected fluorophores. Change the target name as follows:
 - i. FAM: RNaseP
 - ii. CalRed 610: N1
 - iii.: Quasar 670: IAC
 - f. Save the new plate setup for future use
 - i. At the upper left of the screen select “File” then select “Save”
 - iii. Name the file “500003XMPExtract_plateCFX96v2”.
 - iv. Ensure it is saved in the “*.pltd” format
 - v. Select Save
 - vi. Click Ok in the Plate Editor window
 - g. Saving Protocol
 - i. Select “File” then “Open”
 - ii. Select “Protocol” then open your saved protocol.
 - ii. Ensure the PCR steps are correct and click “OK”
 - iii. Click the “Plate” tab and choose “Select Existing”. Choose the plate that was saved.
 - iv. Add any desired notes and click on the “Start Run” tab.
 - v. Save the run with the desired name
 - h. Assigning a sample name to well positions

Once the run has begun, reaction wells can be assigned with a unique sample name as follows:

 - i. Navigate to “Real Time Status”
 - ii. On the top-middle section of the screen, click “Plate Set-Up” followed by “View/Edit Plate”.
 - iii. Click “Spreadsheet View/Importer” on the upper right quadrant of the screen. Wells can be named with unique identifiers for Sample Name, Sample Type, etc.
5. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:
- a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - c. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold

- adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
- d. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
6. Refer to **Table 2** for result interpretation.

APPENDIX D: Bio-Rad CFX384 Touch™ - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **CFX384 Touch™** manual programming specific for Direct Patient sample testing.

1. Launch the CFX Maestro software.
2. Select “File”, then choose “New” and select “Protocol”
3. Program thermocycling conditions in the Protocol Editor
 - a. Set Sample Volume to **20 µL**
 - b. Click “Insert Step” to add a total of five total steps and program as follows (**Table D1**):
 - c. Step 1
 - i. Temp: 46°C
 - ii. Time: 20:00
 - d. Step 2
 - i. Temp: 95°C
 - ii. Time: 5:00
 - e. Step 3
 - i. Temp: 95°C
 - ii. Time: 0:03
 - f. Step 4
 - i. Temp: 62°C
 - ii. Time: 0:20
 - iii. Click “Add Plate Read to Step”. Also, ensure this is the only step containing a plate read
 - g. Step 5
 - i. Temp: 70°C
 - ii. Time: 0:05
 - h. Step 6
 - i. Click to highlight “GOTO” and enter “3” to indicate thermocycling includes Step 3, Step 4 and Step 5.
 - ii. At “GOTO 3”, confirm “39 more times” is programmed for a total of 40 cycles.
 - I. Save the new cycling conditions as protocol for future use
 - i. At the upper left of the screen select the Save button
 - ii. Save in the ExpressLoad folder
 - iii. Name the file ‘500003XMPDirectCFX384’
 - iv. Save as type ‘Protocol File (*.prcl)’
 - v. Select Save
 - vi. Click Ok in the protocol editor window

Table D1. CFX384 Touch™ thermocycling summary - Direct Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Temperature (°C/s)	46	95	95	62	70
Duration (Min:sec)	20:00	5:00	0:03	0:20	0:05
Detection	OFF	OFF	OFF	ON	OFF
No. of PCR Cycles	HOLD	HOLD	40		

4. Define the plate setup

- a. Select “File” then choose “New” and select “Plate”
- b. In the Plate Editor, click settings and ensure your settings are as follows
 - i. Plate Size: 384 well
 - ii. Plate Type: BR White OR BR Clear (BioGX recommends BR White)
- c. Highlight all wells and choose “Select Fluorophores”
 - i. Uncheck all currently selected wells and check ONLY the following: FAM, Cal Red 610, Quasar 670
- d. For “Sample Type” select “Unknown”
- e. Check the boxes besides all three of your selected fluorophores. Change the target name as follows:
 - i. FAM: RNaseP
 - ii. Cal Red 610: N1
 - iii. Quasar 670: IAC
- f. Save the new plate setup for future use
 - i. At the upper left of the screen select “File” then select “Save”
 - iii. Name the file ‘500003XMPDirect_plateCFX384’
 - iv. Save as type ‘Plate File (*.pltd)’
 - v. Select Save
 - vi. Click Ok in the Plate Editor window
- g. Saving Protocol
 - i. Select “File” then “Open”
 - ii. Select “Protocol” then open your saved protocol.
 - ii. Ensure the PCR steps are correct and click “OK”
 - iii. Click the “Plate” tab and choose “Select Existing”. Choose the plate that was saved.
 - iv. Add any desired notes and click on the “Start Run” tab.
 - v. Save the run with the desired name
- h. Assigning a sample name to well positions

- Once the run has begun, reaction wells can be assigned with a unique sample name as follows:
- i. Navigate to “Real Time Status”
 - ii. On the top-middle section of the screen, click “Plate Set-Up” followed by “View/Edit Plate”.
 - iii. Click “Spreadsheet View/Importer” on the upper right quadrant of the screen. Wells can be named with unique identifiers for Sample Name, Sample Type, etc.
5. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:
- a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - d. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
 - e. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
6. Refer to **Table 2** for result interpretation.

Bio-Rad CFX384 Touch™ - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **CFX384 Touch™** manual programming specific for Extracted Patient sample testing.

1. Launch the CFX Maestro software.
2. Choose “File”, then choose “New” and select “Protocol”
3. Program thermocycling conditions in the Protocol Editor
 - a. Set Sample Volume to **15 µL**
 - b. Click “Insert Step” to add a total of five total steps and program as follows (**Table D1**):
 - c. Step 1
 - i. Temp: 46°C
 - ii. Time: 20:00
 - d. Step 2
 - i. Temp: 95°C
 - ii. Time: 5:00
 - e. Step 3
 - i. Temp: 95°C
 - ii. Time: 0:03
 - f. Step 4
 - i. Temp: 62°C
 - ii. Time: 0:20
 - iii. Click “Add Plate Read to Step”. Also, ensure this is the only step containing a plate read
 - g. Step 5
 - i. Temp: 70°C
 - ii. Time: 0:05
 - h. Step 6
 - i. Click to highlight “GOTO” and enter “3” to indicate thermocycling includes Step 3, Step 4 and Step 5.
 - ii. At “GOTO 3”, confirm “39 more times” is programmed for a total of 40 cycles.
 - i. Save the new cycling conditions as a protocol for future use
 - i. At the upper left of the screen select the Save button
 - ii. Save in the ExpressLoad folder
 - iii. Name the file ‘500003XMPExtractCFX384’
 - iv. Save as type ‘Protocol File (*.prcl)’
 - v. Select Save
 - vi. Click Ok in the protocol editor window
4. Define the plate setup
 - a. Select “File” then choose “New” and select “Plate”
 - b. In the Plate Editor, click settings and ensure your settings are as follows
 - i. Plate Size: 384 well
 - ii. Plate Type: BR White OR BR Clear (BioGX recommends BR White)

- c. Highlight all wells and choose “Select Fluorophores”
 - i. Uncheck all currently selected wells and check ONLY the following: FAM, Cal Red 610, Quasar 670
 - d. For “Sample Type” select “Unknown”
 - e. Check the boxes besides all three of your selected fluorophores. Change the target name as follows:
 - i. FAM: RNaseP
 - ii. CalRed 610: N1
 - iii.: Quasar 670: IAC
 - f. Save the new plate setup for future use
 - i. At the upper left of the screen select “File” then select “Save”
 - iii. Name the file “500003XMPExtract_plateCFX384”.
 - iv. Ensure it is saved in the “*.pltd” format
 - v. Select Save
 - vi. Click Ok in the Plate Editor window
 - g. Saving Protocol
 - i. Select “File” then “Open”
 - ii. Select “Protocol” then open your saved protocol.
 - ii. Ensure the PCR steps are correct and click “OK”
 - iii. Click the “Plate” tab and choose “Select Existing”. Choose the plate that was saved.
 - iv. Add any desired notes and click on the “Start Run” tab.
 - v. Save the run with the desired name
 - h. Assigning a sample name to well positions

Once the run has begun, reaction wells can be assigned with a unique sample name as follows:

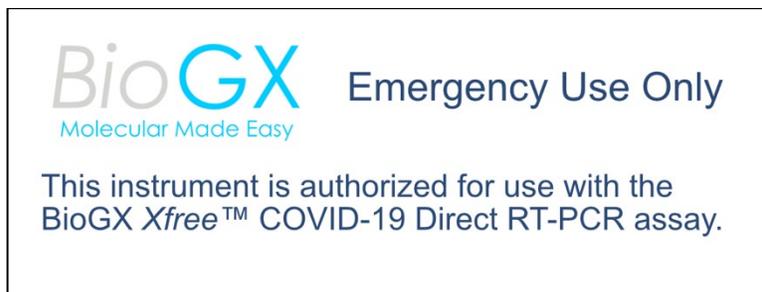
 - i. Navigate to “Real Time Status”
 - ii. On the top-middle section of the screen, click “Plate Set-Up” followed by “View/Edit Plate”.
 - iii. Click “Spreadsheet View/Importer” on the upper right quadrant of the screen. Wells can be named with unique identifiers for Sample Name, Sample Type, etc.
5. When CFX96 Touch™ run is completed, threshold setting update will need to be completed as follows:
- a. Open the “Data Analysis” window for your specific run
 - b. To apply threshold settings for FAM-channel, uncheck CalRed 610-channel and Quasar 670-channel and be sure “**FAM**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
 - i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for FAM will apply to the experiment.
 - d. To apply threshold settings for CalRed 610-channel, uncheck FAM-channel and Quasar 670-channel and be sure “**CalRed 610**” is checked (Note: Threshold

- adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “Baseline Threshold”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for CalRed 610 will apply to the experiment
- e. To apply threshold settings for Quasar 670-channel, uncheck FAM-channel and CalRed 610-channel and be sure “**Quasar 670**” is checked (Note: Threshold adjustments can be performed only in single threshold mode, be sure only one dye is selected)
- i. Navigate to “Settings” and select “**Baseline Threshold**”
 - ii. At the bottom of the popup window under “single threshold”, select “User Defined” and enter “**500**”
 - iii. Click “OK” and the threshold for Quasar 670 will apply to the experiment
6. Refer to **Table 2** for result interpretation.

APPENDIX E: EUO LABELING

For the Applied Biosystems QuantStudio 5, Bio-Rad CFX96 Touch and Bio-Rad CFX384 Touch PCR Instruments

Please print the below label and place the label on the front panel of the instrument. If the instrument includes 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 BioGX Xfree™ COVID-19 Direct RT-PCR assay.



APPENDIX F REVISION HISTORY

Version	Date	Description of Change
Draft 04.1	22 JUN 2021	<p>Addition of Appendix E –EUO Label for platforms</p> <p>Accepted FDA text edit suggestions</p>
Draft 04	14 JUN 2021	<p>FDA redline text suggestions accepted.</p> <p>Added Table of Contents section.</p> <p>Updated Materials Required but not provided section to include pipettes, pipette tips, centrifuge, and 1.5 mL tubes.</p> <p>Added FDA recommended additional warnings and precautions.</p> <p>Updated external positive control run frequency to one external positive control be included with each RT-PCR run of patient samples and external negative controls should be run with each extraction batch (as applicable) and each PCR run.</p> <p>Accepted FDA recommended clinical performance.</p> <p>Specimen collection, storage, and handling section added.</p> <p>Added information for customers on how to obtain EUO label for platforms</p> <p>Minor text edits and formatting</p>
Draft 03.1	26 MAR 2021	<p>Update number of reactions (tests) per tube to number of reactions (tests) per kit. Update Table 2 to simplify interpretation, Revision status updated to 03.1 from 03</p> <p>FDA Recommended Track Changes (28MAY2021)-Update “Preparation of BioGX External Controls” section, update Table. 1 to simplify expected Ct values for N1 and IAC.</p> <p>FDA Recommended Track Changes (08JUN2021)- Update of storage of rehydrated master mix to indicate unused rehydrated master mix is to be discarded</p> <p>Update “Preparation of BioGX External Controls” section to include “Preparation of BioGX External Controls for Direct Sample Workflow”</p> <p>Update “Quality Control” section to include Zeptomatrix SARS-CoV-2 as alternative positive control</p>

		Update dilution protocol in “Preparation of BioGX External Controls” section
03	10 FEB 2021	Update fluorophore utilized for IAC detection, addition of nasopharyngeal wash/aspirates and nasal aspirate sample types, addition of Applied BioSystems 7500 Fast Dx and Bio-Rad CFX384 Touch platforms, update BioGX Rehydration Water Part number to 800-0035-12, update use of Qiagen Viral RNA Mini Kit, update comparator method (Linea™ COVID-19 assay), update clinical concordance study. Revision status updated to 02.4 from 03
02.4	19 NOV 2020	Correction of revision date, “ <i>Validation of this test has not been reviewed by FDA</i> ” paragraph moved to the end of the Intended Use statement on Page 2. Revision status updated to 02.4 from 02.3
02.3	19 NOV 2020	Intended Use statement on Page 2 updated to include “ <i>Validation of this test has not been reviewed by FDA</i> ” paragraph. Revision status updated to 02.3 from 02.2
02.2	18 NOV 2020	Update FDA EUA Review Status statement, Update “Limitations of the Procedure” section, update “Clinical Performance” section, update “Reference” section. Revision status updated to 02.2 from 02.1
02.1	12 NOV 2020	Addition of FDA EUA Review Status, update positive control and patient result interpretation, update limit of detection table, update clinical performance. Revision status updated to 02.1 from 02
02	20 OCT 2020	Correction of BioGX address, minor formatting changes, correction of references, correction of positive control use. Revision status updated to 02 from 01
01	09 OCT 2020	Initial Release

SYMBOLS

Symbol	Meaning
	Catalog number
	<i>In vitro</i> diagnostic medical device
	Do not reuse
	Batch code
	Caution
	Consult instructions for use
	Manufacturer
	Contains sufficient for <n> tests
	Temperature limitation
	Keep dry
	Keep away from sunlight
	Expiration date
	Biological Risks
	Control

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 Technical Support email: TS@biogx.com



Xfree COVID-19 Direct RT-PCR

REF 500-003-XMP
R_x Only IVD

For use under the Emergency Use (EUA) Only

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.

This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.

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.



Be advised, this is not the complete product IFU. Consult current version of Instructions For Use (IFU) and download current version of Electronic User Defined Protocol (eUDP) prior to use. Current versions are available at www.biogx.com OR by emailing info@biogx.com for an electronic version OR a free printed copy.



Current IFU version: **500-003-XMP Xfree™ COVID-19 Direct RT-PCR (US) Rev.Draft03.1**
Current eUDP version:

ABI 7500 Fast Dx:
Direct: 500003XMPDirect7500Dx / 500003XMPDirect7500Dx.sdt
Extracted: 500003XMPEXtract7500Dx / 500003XMPEXtract7500Dx.sdt

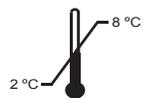
Bio-Rad CFX96 Touch™:
Direct: 500003XMPDirect_plateCFX96v2.pltd / 500003XMPDirectCFX96v2 / 500003XMPDirectCFX96v2.prcl
Extracted: 500003XMPEXtract_plateCFX96v2.pltd / 500003XMPEXtractCFX96v2 / 500003XMPEXtractCFX96v2.prcl

Bio-Rad CFX384 Touch™:
Direct: 500003XMPDirect_plateCFX384.pltd / 500003XMPDirectCFX384 / 500003XMPDirectCFX384.prcl
Extracted: 500003XMPEXtract_plateCFX384.pltd / 500003XMPEXtractCFX384 / 500003XMPEXtractCFX384.prcl

QuantStudio™ 5:
Direct: 500003XMPDirectQS5v2.edt / 500003XMPDirectQS5v2
Extracted: 500003XMPEXtractQS5v2.edt / 500003XMPEXtractQS5v2

<https://biogx.com/xfree/>
Download a free QR Code Scanner App with your mobile phone. Using the QR Code App, take a picture of the QR Code. The App will automatically scan the QR code to obtain current versions of IFU and eUDP via the BioGX website.

STORAGE AND STABILITY



BioGX recommends long-term storage at 2-8°C. Product is stable when shipped for under 5 days at ambient temperature.



Reagents have been tested to demonstrate optimal performance when stored properly at 2-8°C and consumed by the expiration date on the kit label.



Avoid exposing the reagents (lyophilized or rehydrated) to direct sunlight or long-term ambient lighting. Store unused rehydrated master mix up to 24 hours at 2-8°C, protected from light.



If pouch is opened by the user but lyophilized reagent is not rehydrated, avoid exposure of lyophilized reagent to moisture and use the entire contents of the opened pouch within 1 month.

SAFE HANDLING

Use clean gloves when handling PCR reagents and buffer tubes.

Do not use reagent tubes if tube caps have been opened or damaged. Do not mix reagents from different pouches and/or kits and/or lots.

