BioGX
Molecular Made Easy

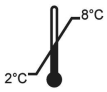
Xfree™ COVID-19 Direct RT-PCR

REF **500-003-XMP**

Σ 104 Tests (Extraction-Free Sample-Direct Tests)

Instructions For Use

For *In Vitro* Diagnostic Use

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500-003-XMP (CE-IVD) Rev. 09-07APR2023

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PROPRIETARY NAME

Xfree™ COVID-19 Direct RT-PCR

INTENDED USE

The BioGX *Xfree*™ COVID-19 Direct RT-PCR ("*Xfree*™ COVID-19") is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 virus in specimens collected in Copan UTM®, Copan ESwab™, BD UVT™, VTM or saline (0.85% NaCl) using nasopharyngeal, nasal, mid-turbinate, and oropharyngeal swab, or nasopharyngeal wash/aspirates or nasal aspirates obtained from individuals suspected of COVID-19 by their healthcare provider.

Testing has been validated for analysis of:
a.) direct addition of individual patient sample
b.) extracted nucleic acid addition of individual patient sample
c.) direct addition of pooled patient sample

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in nasopharyngeal, nasal, mid-turbinate, and oropharyngeal swab specimens, nasopharyngeal wash/aspirates or nasal aspirates 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. 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 performance of this test was established based on the evaluation of a limited number of clinical specimens. Clinical performance has not been established with 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 is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR *in vitro* diagnostic procedures and use of the following real-time PCR instruments:

-**pixl™ Real-Time PCR Platform** (software version 1.6.9.0 or later)
-**Applied Biosystems™ QuantStudio™ 5** (0.2 mL 96-well; Design & Analysis software version 1.5.1 or later)
-**Applied Biosystems™ QuantStudio™ 5**, 384-well (Design & Analysis software version 1.5.1 or later)
-**Applied Biosystems™ 7500 Fast Dx** (SDS software version 1.4 or later)
-**Bio-Rad CFX96 Touch™** (Maestro™ software version 1.1 or later)
-**Bio-Rad CFX384 Touch™** (Maestro™ software version 1.1 or later)
-**Bio Molecular Systems Mic Instrument** (Mic PCR software version 2.8 or later)

One format of the BioGX *Xfree*™ COVID-19 assay kit is available:

1. BioGX *Xfree*™ COVID-19 lyophilized format (**REF: 500-003-XMP**)

The BioGX *Xfree*™ COVID-19 lyophilized format assay has been validated utilizing two different workflows, the direct workflow in which patient sample is added directly to the BioGX RT-PCR master 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. The *Xfree*™ COVID-19 assay has CE-IVD marking.

SUMMARY AND EXPLANATION

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 death[1].

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 transfer[3].

As of April 12, 2022, the WHO has confirmed 497,057,239 infections and 6,179,104 COVID-19 related deaths globally[4]. 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[5].

BioGX *Xfree*™ COVID-19 utilizes primer and probe sets based on the United States Centers for Disease Control and Prevention (US CDC) assay for specific detection of SARS-CoV-2 (N1 gene region) and human RNase P[1,2,6] that serves as an endogenous Sample Processing Control. Additionally, a non-naturally occurring single-stranded RNA has been integrated into the master mix to serve as an internal amplification control.

The BioGX *Xfree*™ COVID-19 multiplex assay is provided in BioGX Sample-Ready™ lyophilized format (REF: 500-003-XMP) within 4 x 2 mL vials. Each vial contains all PCR components such as primers, probes, enzymes, dNTPs, $MgCl_2$, Internal Amplification Control RNA (IAC), and buffers required for real-time RT-PCR-based testing.

Individual Patient Samples

BioGX *Xfree*™ COVID-19 kit (REF: 500-003-XMP) supports testing of individual patient samples for 104 direct patient samples or 160 extracted/purified nucleic acid samples. Direct and extracted patient sample analysis has been validated with the following platforms:

-**Applied Biosystems™ QuantStudio™ 5** (0.2 mL 96-well; Design & Analysis software version 1.5.1 or later)
-**Applied Biosystems™ QuantStudio™ 5** (384-well**;** Design & Analysis software version 1.5.1 or later)
-**Applied Biosystems™ 7500 Fast Dx** (SDS software version 1.4.1)
-**Bio-Rad CFX96 Touch™** (Maestro™ software version 1.1 or later)
-**Bio-Rad CFX384 Touch™** (Maestro™ software version 1.1 or later)
-**Bio Molecular Systems Mic PCR Instrument** (Mic PCR software version 2.8 or later)
-**pixl™ Real-Time PCR Platform** (software version 1.6.9.0 or later)

Pooled Patient Samples

BioGX *Xfree*™ COVID-19 kit (REF: 500-003-XMP) supports testing of 52 pooled samples (52 x 5 patient sample pools per PCR reaction). Pooled sample analysis has been validated with the following platforms:

-**Applied Biosystems™ QuantStudio™ 5** (0.2 mL 96-well; Design & Analysis software version 1.5.1 or later)
-**Applied Biosystems™ 7500 Fast Dx** (SDS software version 1.4.1)
-**Bio-Rad CFX96 Touch™** (Maestro™ software version 1.1 or later)
-**pixl™ Real-Time PCR Platform** (software version 1.6.9.0 or later)

Validated Nasopharyngeal and Oropharyngeal Specimen Collection Devices

The following sample types and real-time PCR platforms have been validated for **individual direct patient sample** addition when using **BioGX *Xfree*™ COVID-19 lyophilized format**:

- **Applied Biosystems™ Quantstudio™ 5 (0.2mL 96-well), Applied Biosystems™ Quantstudio™ 5 (384-well), Applied Biosystems™ 7500 Fast DX, Bio-Rad CFX96 Touch™, Bio-Rad CFX384 Touch™, Bio Molecular Mic platform**
  - Specimen Collections in:
    - Copan ESwab
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))
- **pixl™ Real-Time PCR Platform**
  - Specimen Collections in:
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))

The following sample types and real-time PCR platforms have been validated for **pooled direct patient sample** addition when using **BioGX *Xfree*™ COVID-19 lyophilized format**:

- **Applied Biosystems™ Quantstudio™ 5 (0.2mL 96-well), Applied Biosystems™ 7500 Fast DX, Bio-Rad CFX96 Touch™**
  - Specimen Collections in:
    - Copan ESwab
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))
- **pixl™ Real-Time PCR Platform**
  - Specimen Collections in:
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))

The following sample types and real-time PCR platforms have been validated for **nucleic acid extracted individual sample** addition when using **BioGX *Xfree*™ COVID-19 lyophilized format**:

- **Applied Biosystems™ Quantstudio™ 5 (0.2mL 96-well), Applied Biosystems™ Quantstudio™ 5 (384-well), Applied Biosystems™ 7500 Fast DX, Bio-Rad CFX96 Touch™, Bio-Rad CFX384 Touch™, Bio Molecular Mic platform**
  - Specimen Collections in:
    - Copan ESwab
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))
- **pixl™ Real-Time PCR Platform**
  - Specimen Collections in:
    - Copan Universal Transport Media (UTM)
    - BD Universal Viral Transport (UVT)
    - Viral Transport Media (VTM)
    - Dry swab (resuspended in saline (0.85% NaCl))

PRINCIPLES OF THE PROCEDURE

The BioGX *Xfree*™ COVID-19 is to be used with the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well and 384-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System Bio-Rad CFX384 Touch™ Real-Time PCR Detection System, Bio Molecular Systems Mic, or pixl™ Real-Time PCR Platform 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 amount of fluorescence detected in the three optical channels used for the BioGX *Xfree*™ COVID-19 is directly proportional to the quantity of the corresponding probe that is hydrolyzed, and therefore generally proportional to the amount of synthesized target. The Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well and 384-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad CFX384 Touch™ Real-Time PCR Detection System, Bio Molecular Systems Mic, or pixl™ Real-Time PCR Platform 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 PROVIDED

Qty	REF	Contents	No. Tests Direct Format	No. Tests Extracted Format	No. Tests Pooled Format
1	500-003-XMP	**BioGX *Xfree*™ COVID-19 Direct RT-PCR Extraction-Free - Multi-Platform** Sample-Ready™ lyophilized PCR Master Mix containing polymerase, reverse transcriptase, primers, probes, dNTPs, $MgCl_2$, Internal Amplification Control RNA (IAC), and buffers	104	160	52

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) (BioGX catalog no. 720-0206).
- Sterile DNase/RNase free 1.5 mL snap cap tubes.
- BioGX Molecular Grade Water or equivalent.
  - BioGX Rehydration Water (BioGX catalog number: 800-0035-12)
- Sterile swab collection device appropriate for nasal, nasopharyngeal 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.
- Optional if using extraction
  - QIAGEN QIAamp Viral RNA Mini Kit (Qiagen catalog no. 52906)
  - Centrifuge compatible with QIAGEN extraction kit tubes
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well) consumables.
  - Thermo Fisher optical 8-tube clear strip (Thermo Fisher catalog no. 4316567)
  - Thermo Fisher ultra-clear optical caps, strips of 8 (Thermo Fisher catalog no. AB-0866)
  - Thermo Fisher 96-well optical clear reaction plates (Thermo Fisher catalog no. A36924)
  - Thermo Fisher 96-well qPCR plate seals (Thermo Fisher catalog no. AB-1170)
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well) consumables.
  - Thermo Fisher MicroAmp® Endura Plate Optical 384-well Clear Reaction Plate with Barcode (Thermo Fisher catalog no.: 4483258 or equivalent)
  - Thermo Fisher Optical plate seal (Thermo Fisher catalog no. AB0558 or equivalent)
- Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (0.1 mL) consumables.
  - Thermo Fisher optical 8-tube clear strip (Thermo Fisher catalog no. 4358293)

  - Thermo Fisher ultra-clear optical caps, strips of 8 (Thermo Fisher catalog no. 4323032)
  - Thermo Fisher 96-well optical reaction plates (Thermo Fisher catalog no. 4346906)
  - Thermo Fisher 96-well qPCR plate seals (Thermo Fisher catalog no. 4311971)
- Bio-Rad CFX96 Touch™ Real-Time PCR Detection System consumables.
  - Bio-Rad 8-tube white PCR strips without caps (Bio-Rad catalog no. TLS0851)
  - Bio-Rad Optical flat 8-cap strips for PCR tubes (Bio-Rad catalog no. TCS0803)
  - Bio-Rad 96-well white plates (Bio-Rad catalog no. HSP9655)
  - Bio-Rad 96-well plate sealing film, optical (Bio-Rad catalog no. MSB1001)
- Bio-Rad CFX384 Touch™ Real-Time PCR Detection System consumables.
  - Bio-Rad 384-well clear shell/white well plates (Bio-Rad catalog no. HSP3905)
  - Bio-Rad 384-well plate sealing film, optical (Bio-Rad catalog no. MSB1001)
- Bio Molecular Systems Mic PCR Instrument consumables
  - Mic PCR Instrument clear reaction tubes and caps (Bio Molecular Systems catalog no. 60653)
  - Mic PCR Instrument reaction tubes loading block (Bio Molecular Systems catalog no. 80418)
  - Mic PCR Instrument reaction tubes capping tool (Bio Molecular Systems catalog no. 90690)
- PCR instruments: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well and 384-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad CFX384 Touch™ Real-Time PCR Detection System, Bio Molecular Systems Mic, or pixl™ Real-Time PCR Platform
- pixl™ Real-Time PCR Platform (BioGX catalog no. 650-016-PXL) Instrument consumables
  - TempAssure® PCR 8-Tube clear strips, Att. Optical Caps (BioGX catalog no. 010-280-ETS)
- Multi-well Plate Centrifuge (Fisher Mini Plate Spinner catalog no. 14-100-143) or equivalent

WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use.
- 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.
- 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 M29[7] and in Biosafety in Microbiological and Biomedical Laboratories[8].
- Performance characteristics of this test have been established only with the specimen types listed in the “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.
- Avoid exposing reagents (lyophilized or rehydrated) to direct sunlight of long-term ambient lighting.

STORAGE AND STABILITY

- BioGX recommends long-term storage at 2 to 8°C. Product is stable at a temperature range of 2-30°C during shipment for 5 days.
- 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.
- Discard unused rehydrated master mix if not used immediately. Re-use may result in contamination and false results.
- If the 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.

SPECIMEN COLLECTION, STORAGE, AND HANDLING

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

Collection: 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, UVT, VTM) or 1 mL of modified liquid Amies media (ESwab).

- Nasopharyngeal swab (NP): Insert a swab into the nostril parallel to the palate. Swab should reach a depth equal to the distance from the nostrils to the outer opening of the ear. Leave the swab in place for several seconds to absorb secretions. Slowly remove the swab while rotating it.
- Oropharyngeal swab (e.g., throat swab, OP): Swab the posterior pharynx, avoiding the tongue.
- Anterior Nasal Swab (NS): Using a flocked 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 the 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 an 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

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)
(Operating software: Design & Analysis Software v1.5.1 or later)

Install the BioGX Protocol on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)
It will be necessary to import the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System template. The most current Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System 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. Depending on your geographical location, select "U.S. Product Documents" or "Int. 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 Real-Time PCR System template. Alternatively, the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System template can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix A**. For further instrument information, please refer to the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System user manual[9].

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well)
(Operating software: Design & Analysis Software v1.5.1 or later)

Install the BioGX Protocol on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well
It will be necessary to import the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well template. The most current Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well 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. Depending on your geographical location, select "U.S. Product Documents" or "Int. 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 Real-Time PCR System, 384-well template. Alternatively, the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well template can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix B**. For further instrument information, please refer to the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well user manual[9].

Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument
(Operating software: SDS Software v1.4.1)

Install the BioGX Protocol on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument
It will be necessary to import the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument template. The most current Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument 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. Depending on your geographical location, select "U.S. Product Documents" or "Int. 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 Real-Time PCR Instrument template can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix C**. For further instrument information, please refer to the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument user manual[10].

Bio-Rad CFX96 Touch™ Real-Time PCR Detection System
(Operating software: Maestro 1.1 or later)

Install the BioGX Protocol on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System
It will be necessary to import the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System Protocol and Plate files. The most current versions of the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System 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. Depending on your location, select "U.S. Product Documents" or "Int. 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™ Real-Time PCR Detection System Protocol and Plate files. Alternatively, the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System Protocol and Plate files can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix D**. For further instrument information, please refer to the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System user manual[11].

Bio-Rad CFX384 Touch™ Real-Time PCR Detection System
(Operating software: Maestro 1.1 or later)

Install the BioGX Protocol on the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System
It will be necessary to import the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System Protocol and Plate files. The most current versions of the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System 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. Depending on your location, select "U.S. Product Documents" or "Int. 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™ Real-Time PCR Detection System Protocol and Plate files. Alternatively, the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System Protocol and Plate files can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix E**. For further instrument information, please refer to the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System user manual[11].

pixl™ Real-Time PCR Platform
(Operating software: version 1.6.9.0 or later)

Install the BioGX Protocol on the pixl™ Real-Time PCR Platform. It will be necessary to import the pixl™ Real-Time PCR Platform template. The most current versions of the pixl™ Real-Time PCR Platform template are available for download on **www.biogx.com.** Follow the "Education Center" tab in the navigation menu at the top of the home page. Depending on your location, select "U.S. Product Documents" or "Int. 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 pixl™ Real-Time PCR Platform template. Alternatively, the pixl™ Real-Time PCR Platform template can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix F**. For further instrument information, please refer to the pixl™ Real-Time PCR Platform user manual[12].

Bio Molecular Systems Mic Instrument
(Operating software: version 2.8 or later)

Install the BioGX Protocol on the Mic Instrument
It will be necessary to import the Mic Instrument template. The most current versions of the Mic Instrument template are available for download on **www.biogx.com.** Follow the "Education Center" tab in the navigation menu at the top of the home page. Depending on your location, select "U.S. Product Documents" or "Int. 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 Mic Instrument. Alternatively, the Mic Instrument template can also be obtained by emailing BioGX at **info@biogx.com** or **eu@biogx.com**. To manually program the thermocycler, please refer to **Appendix G**. For further instrument information, please refer to the Bio Molecular Systems Mic Instrument user manual[13].

SPECIMEN PREPARATION

Direct Individual Patient Sample Addition

Pipette **5 µL** of patient sample directly to tube/well containing RT-PCR master mix.

Validated sample types include nasopharyngeal, nasal, mid-turbinate, and oropharyngeal swabs collected in 1 mL collection media or 3 mL collection media. Additional validated sample types include nasopharyngeal wash/aspirates or nasal aspirate collected in saline (0.85% NaCl).

Follow the "RT-PCR Set-up - Direct Patient Sample" section below for the appropriate real-time PCR platform (i.e. Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well), Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad CFX384 Touch™ Real-Time PCR Detection System, Mic Instrument and pixl™ Real-Time PCR Platform).

Extracted Individual Patient Sample Addition

Pipette **5 µL** of purified nucleic acid eluate directly into a tube/well containing RT-PCR master mix.

Validated sample types extracted with Qiagen Viral RNA Mini Kit include nasopharyngeal, nasal, mid-turbinate, and oropharyngeal swabs collected in 1 mL collection media or 3 mL collection media.

Follow the "RT-PCR Set-up - Extracted Patient Sample" section below for the appropriate real-time PCR platform (i.e. Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well), Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad CFX384 Touch™ Real-Time PCR Detection System, Mic Instrument and pixl™ Real-Time PCR Platform). For validated magnetic bead or silica column nucleic acid extraction method, pipette appropriate volume of specimen into the extraction tube/plate and proceed with protocol as per manufacturer's instructions for use. After extraction and elution, add **5 µL** of the eluate into a tube/well containing RT-PCR master mix.

Pooled Direct Patient Sample Addition

Pipette **10 µL** of pooled patient sample (equal volumes of up to 5 patient samples can be pooled) directly to the tube/well containing RT-PCR master mix.

Validated sample types include nasopharyngeal, nasal, mid-turbinate, and oropharyngeal swabs collected in 1 mL collection media or 3 mL collection media. Additional validated sample types include nasopharyngeal wash/aspirates or nasal aspirate collected in saline (0.85% NaCl).

Follow the "RT-PCR Set-up - Pooled Direct Patient Sample" section below for the appropriate real-time PCR platform (i.e. Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and pixl™ Real-Time PCR Platform.

Other Sample Types

This assay has been optimized for use with the specimen types and volumes described above. Use of any other specimen type, collection method, or sample volume may be inhibitory to the PCR. BioGX does not make claims for processing methods or sample types other than those described in this manual.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System,
(0.2 mL 96-well)

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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 is not fully dispensed, discard unused volume).
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 instrument platform appropriate optical caps or optical plate seals.
8. Pulse spin the sealed 8-tube PCR strips or 96-well PCR plate to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips 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 Real-Time PCR System 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 Real-Time PCR System** for download instructions. To manually program the thermocycler, please refer to **Appendix A**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well)

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 is not fully dispensed, discard unused volume).
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 instrument platform appropriate optical caps or optical plate seals.
8. Pulse spin the sealed PCR plate to mix and bring liquid to the bottom.
9. Load 384-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 Real-Time PCR System, 384-well template file for Direct Patient Sample addition (500003XMPDirectQS5_384Rev01.edt), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well)** for download instructions. To manually program the thermocycler, please refer to **Appendix B**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument

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 is not fully dispensed, discard unused volume).
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 instrument platform appropriate optical caps or optical plate seals.
8. Pulse spin the sealed 8-tube PCR strips or 96-well PCR plate to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips 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 Real-Time PCR Instrument 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 Real-Time PCR Instrument** for download instructions. To manually program the thermocycler, please refer to **Appendix C**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

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 is not fully dispensed, discard unused volume).
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 8-tube PCR strips or 96-well PCR plates to mix and bring liquid to the bottom.
9. When the CFX96 Touch™ run is completed, the 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™ Real-Time PCR Detection System 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™ Real-Time PCR Detection System** for download instructions. To manually program the thermocycler, please refer to **Appendix D**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Bio-Rad CFX384 Touch™ Real-Time PCR Detection System

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 is not fully dispensed, discard unused volume).
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 384-well PCR plate 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™ Real-Time PCR Detection System 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™ Real-Time PCR Detection System** for download instructions. To manually program the thermocycler, please refer to **Appendix E**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: pixl™ Real-Time PCR Platform

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.
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 is not fully dispensed, discard unused volume).
5. Transfer **15 µL** of **rehydrated master mix** to the bottom of **1 to 16 empty PCR tubes** (8-tube PCR strips).
6. To each PCR tube containing 15 µL of rehydrated master mix, add **5 µL** of **direct patient sample**.
7. Affix the optical caps.
8. Pulse spin the sealed 8-tube PCR strips to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips into the real-time PCR platform and start run. Avoid unnecessary delay once tubes are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For pixl™ Real-Time PCR Platform template file for Direct Patient Sample addition (500003XMPDirExtpixlRev01.json), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the pixl™ Real-Time PCR Platform** for download instructions. To manually program the thermocycler, please refer to **Appendix F**.

RT-PCR SET-UP - DIRECT PATIENT SAMPLE: Bio Molecular Systems Mic Instrument

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 PCR tubes.
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 is not fully dispensed, discard unused volume).
5. Transfer **15 µL** of **rehydrated master mix** to the bottom of **26 empty PCR tubes.** To each PCR tube containing 15 µL of rehydrated master mix, add **5 µL** of **direct patient sample**.
6. Affix the reaction tube caps.
7. Pulse spin the sealed PCR tubes to mix and bring liquid to the bottom.
8. Load the reaction tubes into the real-time PCR platform and start run. Avoid unnecessary delay once tubes are loaded.
9. Refer to **Table 2** for result interpretation.

Note: For Bio Molecular Systems Mic Instrument template file for Direct Patient Sample addition (500003XMPDirectMIC.mictemplate), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the Bio Molecular Systems Mic Instrument** for download instructions. To manually program the thermocycler, please refer to **Appendix G**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)

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 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 is not fully dispensed, discard unused volume).
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 8-tube PCR strips or 96-well PCR plate to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips 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 Real-Time PCR System 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 Real-Time PCR System** for download instructions. To manually program the thermocycler, please refer to **Appendix A**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (384-well)

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 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 is not fully dispensed, discard unused volume).
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 **extracted patient sample**.
7. Affix the appropriate optical plate seals.
8. Pulse spin the sealed 384-well PCR plates to mix and bring liquid to the bottom.
9. Load 384-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once plates are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well template file for Extracted Patient Sample addition (500003XMPExtractedQS5_384Rev01.edt), please refer to the section: "**Instrument Templates and Programming**": **Install the BioGX Protocol on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well** for download instructions. To manually program the thermocycler, please refer to **Appendix B**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument

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 is not fully dispensed, discard unused volume).
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 8-tube PCR strips or 96-well PCR plates to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips 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 Real-Time PCR Instrument 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 Real-Time PCR Instrument** for download instructions. To manually program the thermocycler, please refer to **Appendix C**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

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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 is not fully dispensed, discard unused volume).
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. 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™ Real-Time PCR Detection System 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™ Real-Time PCR Detection System** for download instructions. To manually program the thermocycler, please refer to **Appendix D**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Bio-Rad CFX384 Touch™ Real-Time PCR Detection System

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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 is not fully dispensed, discard unused volume).
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 **extracted patient sample**.
7. Affix the appropriate optical plate seals.
8. Pulse spin the sealed 384-well PCR plate 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™ Real-Time PCR Detection System 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™ Real-Time PCR Detection System** for download instructions. To manually program the thermocycler, please refer to **Appendix E**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: pixl™ Real-Time PCR Platform

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.
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 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 is not fully dispensed, discard unused volume).
5. Transfer **10 µL** of **rehydrated master mix** to the bottom of **1 to 16 empty wells** (8-tube PCR strips).
6. To each well containing 10 µL of rehydrated master mix, add **5 µL** of **extracted patient sample**.
7. Affix the optical caps.
8. Pulse spin the sealed tubes to mix and bring liquid to the bottom.
9. Load 8-tube PCR strips into the real-time PCR platform and start run. Avoid unnecessary delay once tubes are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For pixl™ Real-Time PCR Platform template file for Extracted Patient Sample addition (500003XMPDirExtpixlRev01.json), please refer to the section: "**Instrument Templates and Programming**": **Install the BioGX Protocol on the pixl™ Real-Time PCR Platform** for download instructions. To manually program the thermocycler, refer to **Appendix F**.

RT-PCR SET-UP - EXTRACTED PATIENT SAMPLE: Bio Molecular Systems Mic Instrument

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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 PCR tubes.
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 is not fully dispensed, discard unused volume).
5. Transfer **10 µL** of **rehydrated master mix** to the bottom of **40 empty wells** (PCR tubes).
6. To each well containing 10 µL of rehydrated master mix, add **5 µL** of **extracted patient sample**.
7. Affix the reaction tube caps.
8. Pulse spin the sealed PCR tubes to mix and bring liquid to the bottom.
9. Load the reaction tubes into the real-time PCR platform and start run. Avoid unnecessary delay once tubes are loaded.
10. Refer to **Table 2** for result interpretation.

Note: For Bio Molecular Systems Mic Instrument template file for extracted Patient Sample addition (500003XMPExtractedMIC.mictemplate), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the Bio Molecular Systems Mic Instrument** for download instructions. To manually program the thermocycler, please refer to **Appendix G**.

RT-PCR SET-UP - POOLED DIRECT PATIENT SAMPLE: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)

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 13 pooled samples (each pooled sample consisting of 5 individual patient 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 is not fully dispensed, discard unused volume).
5. Transfer **30 µL** of **rehydrated master mix** to the bottom of **13 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. Prepare one pool of 5 patient samples by transferring an equal volume (minimum of **50 µL**) of each patient sample into an empty sterile tube. Mix by gently pipetting up and down.
7. To each well containing 30 µL of rehydrated master mix, add **10 µL** of **pooled direct patient sample**.
8. Affix the appropriate optical caps or optical plate seals.
9. Pulse spin the sealed 8-tube PCR strips or 96-well PCR plates to mix and bring liquid to the bottom.
10. Load 8-tube PCR strips or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
11. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System template file for Pooled Direct Patient Sample addition (500003XMPDirectPooledQS5Rev01.edt), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System** for download instructions. To manually program the thermocycler, please refer to **Appendix A**.

RT-PCR SET-UP - POOLED DIRECT PATIENT SAMPLE: Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument

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 is not fully dispensed, discard unused volume).
5. Transfer **30 µL** of **rehydrated master mix** to the bottom of **13 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. Prepare one pool of 5 patient samples by transferring an equal volume (minimum of **50 µL**) of each patient sample into an empty sterile tube. Mix by gently pipetting up and down.
7. To each well containing 30 µL of rehydrated master mix, add **10 µL** of pooled **direct patient sample**.
8. Affix the instrument platform appropriate optical caps or optical plate seals.
9. Pulse spin the sealed 8-tube PCR strips or 96-well PCR plate to mix and bring liquid to the bottom.
10. Load 8-tube PCR strips or 96-well PCR plates into the real-time PCR platform and start run. Avoid unnecessary delay once tubes/plates are loaded.
11. Refer to **Table 2** for result interpretation.

Note: For Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument template file for Pooled Direct Patient Sample addition (500003XMPDirectPooled7500DxRev01.sdt), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument** for download instructions. To manually program the thermocycler, please refer to **Appendix C**.

RT-PCR SET-UP - POOLED DIRECT PATIENT SAMPLE: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

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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 13 pooled samples (each pooled sample consisting of 5 individual patient 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 is not fully dispensed, discard unused volume).
5. Transfer **30 µL** of **rehydrated master mix** to the bottom of **13 empty wells** (8-tube PCR strips or 96-well PCR plate).
6. Prepare a pool of 5 patient samples by transferring an equal volume (minimum of **50 µL**) of each patient sample into an empty sterile tube. Mix by gently pipetting up and down.
7. To each well containing 30 µL of rehydrated master mix, add **10 µL** of the **pooled direct patient sample**.
8. Affix the appropriate optical caps or optical plate seals.
9. Pulse spin the sealed 8-tube PCR strips or 96-well PCR plates to mix and bring liquid to the bottom.
10. 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

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

Note: For Bio-Rad CFX96 Touch™ Real-Time PCR Detection System protocol and plate files for Pooled Direct Patient Sample addition (500003XMPDirectPooledCFX96Rev01.prcl and 500003XMPDirectPooledCFX96PlateRev01.pltd), please refer to the section: **“Instrument Templates and Programming”**: **Install the BioGX Protocol on the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System** for download instructions. To manually program the thermocycler, please refer to **Appendix D**.

RT-PCR SET-UP - POOLED DIRECT PATIENT SAMPLE: pixl™ Real-Time PCR Platform

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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.
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 13 pooled samples (each pooled sample consisting of 5 individual patient 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 is not fully dispensed, discard unused volume).
5. Transfer **30 µL** of **rehydrated master mix** to the bottom of **13 empty wells** (8-tube PCR strips).
6. Prepare one pool of 5 patient samples by transferring an equal volume (minimum of **50 µL**) of each patient sample into an empty sterile tube. Mix by gently pipetting up and down.
7. To each well containing 30 µL of rehydrated master mix, add **10 µL** of **pooled direct patient sample**.
8. Affix the appropriate optical caps.
9. Pulse spin the sealed 8-tube PCR strips to mix and bring liquid to the bottom.
10. Load 8-tube PCR strips into the real-time PCR platform and start run. Avoid unnecessary delay once tubes are loaded.
11. Refer to **Table 2** for result interpretation.

Note: For pixl™ Real-Time PCR Platform template file for Pooled Direct Patient Sample addition (500003XMPDirExtpixlRev01.json), please refer to the section: **"Instrument Templates and Programming"**: **Install the BioGX Protocol on the pixl™ Real-Time PCR Platform** for download instructions. To manually program the thermocycler, refer to **Appendix F**.

QUALITY CONTROL

CONTROL

Each BioGX *Xfree*™ COVID-19 includes molecular primers and probes specific for the detection of the endogenous extraction control, human RNase P gene. RNase P should be present and detected in each patient sample. A non-naturally occurring single-stranded RNA integrated into the lyophilized reagent serves 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 Sample Processing Control (SPC) or Internal Amplification Control (IAC) is required.

Laboratories must establish the number, type, and frequency of testing of control materials according to guidelines or requirements of local, provincial, state, and federal and/or country regulations or accreditation organizations in order to monitor the effectiveness of the entire analytical process. It is generally recommended to run appropriate positive and negative controls with every sample extraction/PCR run. 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. For general quality control guidance, the user may wish to refer to CLSI MM3[14] and EP12[15]. The External Positive Control is intended to monitor for substantial reagent failure. The External Negative Control is intended to detect reagent or environmental contamination (or carry-over) by target nucleic acids.

External positive and negative controls are available separately for the BioGX *Xfree*™ COVID-19 kit. The External Control available from BioGX (BioGX Synthetic Nucleocapsid Phosphoprotein gene region (N1) (BioGX catalog no. 720-0206) is treated as if it were a patient sample. Refer to **Tables 1A and 1B** in the “Results Interpretation” section for the interpretation of External Control assay results. 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.

Preparation of BioGX External Control for Direct or Extracted Sample Testing

Applied Biosystems™, Bio-Rad, or Bio Molecular Systems platforms

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/µL).

1. Rehydrate one BioGX lyophilized SARS-CoV-2 (N1) control bead with 100 µL 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 µL 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 µL 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 µL 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 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.

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

All External Controls should yield the expected results (**Table 1A**). An External Negative Control that yields a positive result is indicative of errant sample handling and/or contamination. An External Positive Control that yields a negative result is indicative of a specimen handling/preparation problem. Review the specimen handling/preparation technique.

An External Control that yields an Unresolved, Indeterminate, or Incomplete test result is indicative of a reagent or a real-time PCR system failure. Check the platforms for any error messages. Refer to the "Troubleshooting" section of the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)[9], Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well[9], Applied Biosystems™ 7500 Fast Dx[10] Real-Time PCR Instrument, Bio-Rad CFX96 Touch™/CFX384 Touch™ [11] Real-Time PCR Detection System and Bio Molecular Mic System[13] Instruction Manuals for interpretation of warning and error codes. If the problem persists, use reagents from an unopened pouch or use a new assay kit.

For further reference, please reference the product insert for **Lyophilized Control Template Beads (BioGX Product Number Series 720-XXXX)** 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 depending on your location select "Int. Product Documents" or "U.S. Product Documents". Choose the appropriate product number under "Template Controls".

Table 1A. Interpretation of BioGX external controls when run on **Applied Biosystems™, Bio-Rad, or Bio Molecular Systems platforms.**

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

Preparation of BioGX External Controls - pixl™ Real-Time PCR Platform

Positive Control [BioGX SARS-CoV-2 (N1)] - Each lyophilized template bead should be rehydrated with 500 µL of molecular grade water and mixed by pipetting up and down 5 times. Add **5 µL** of the positive control to (1) well position containing dispensed master mix.

Negative Control (No Template) - Addition of **5 µL** of molecular grade water to (1) well position containing dispensed master mix.

All External Controls should yield the expected results (**Table 1B**). An External Negative Control that yields a positive result is indicative of errant sample handling and/or contamination. An External Positive Control that yields a negative result is indicative of a specimen handling/preparation problem. Review the specimen handling/preparation technique.

An External Control that yields an Unresolved, Indeterminate, or Incomplete test result is indicative of a reagent or a real-time PCR system failure. Check the pixl™ Real-Time PCR Platform for any error messages. Refer to the "Troubleshooting" section of the pixl™ Real-Time PCR Platform Instruction for Use Manual[12] for interpretation of warning and error codes. If the problem persists, use reagents from an unopened pouch or use a new assay kit.

For further reference, please reference the product insert for **Lyophilized Control Template Beads (BioGX Product Number Series 720-XXXX)** 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 depending on your location select "Int. Product Documents" or "U.S. Product Documents". Choose the appropriate product number under "Template Controls".

Table 1B. Interpretation of BioGX external controls when run on **pixl™ Real-Time PCR Platform**.

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

Preparation of ZeptoMetrix External Positive Control for Extracted or Direct Samples
Preparation of ZeptoMetrix SARS-Related Coronavirus 2 (SARS-CoV-2 Isolate: USA-WA1/2020) ZeptoMetrix catalog number: NATSARS(COV2)-ST

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

1. With a fresh pipette tip, prepare **two** microcentrifuge tubes for serial dilution by adding 18 µL 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 /µL and 10 copies / µL, respectively.
2. With a fresh pipette tip, transfer 2 µL 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 µL 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 ZeptoMetrix 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.

RESULTS INTERPRETATION

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)

Results are available under the "Results" tab in the QuantStudio Design and Analysis Software. To download the data, the user can navigate to "Export" tab and export the data in a variety of formats (*.xls, *.xlsx, and *.txt). Alternatively, the user can navigate to "Print report" under "File" tab to preview and save the report in *.PDF format. Please refer to the QuantStudio™ 5 Real-Time PCR System user manual for further instructions[9].

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well

Results are available under the "Results" tab in the QuantStudio Design and Analysis Software. To download the data, the user can navigate to "Export" tab and export the data in a variety of formats (*.xls, *.xlsx, and *.txt). Alternatively, the user can navigate to "Print report" under "File" tab to preview and save the report in *.PDF format. Please refer to the QuantStudio™ 5 Real-Time PCR System, 384-well user manual for further instructions[9].

Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument

Results are available under the "Results" tab in the SDS Software. To download the data, the user can navigate to "File" then click "Export". Choose "Results", select a desired file name to save, and check both boxes on the "Export Settings" pop-up to export the results as a *.csv. Alternatively, the user can save a PDF of the run report by navigating to the "Report" section of the "Results" tab and selecting the print icon. Please refer to the 7500 Fast Dx Real-Time PCR Instrument user manual for further instructions[10].

Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

Results are available to view and download in pdf format by navigating to the "Tools" tab and selecting the "Report" option on the Bio-Rad CFX Maestro software in Data Analysis mode. Alternatively, the user can navigate to the "Export" tab and export all raw data sheets in a variety of formats (*.xls, *.xlsx, and *.txt). Please refer to the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System user manual for further instructions[11].

Bio-Rad CFX384 Touch™ Real-Time PCR Detection System

Results are available to view and download in pdf format by navigating to the "Tools" tab and selecting the "Report" option on the Bio-Rad CFX Maestro software in Data Analysis mode. Alternatively, the user can navigate to the "Export" tab and export all raw data sheets in a variety of formats (*.xls, *.xlsx, and *.txt). Please refer to the Bio-Rad CFX384 Touch™ Real-Time PCR Detection System user manual for further instructions[11].

pixl™ Real-Time PCR Platform

Results are available to view and download in .pdf format by navigating to the "Results" tab, choosing the appropriate experiment, and selecting the "Export" option on the pixl™ Real-Time PCR Platform software. Results are also available to download in the following formats: *.csv, *.excel, *.temp, *.json, *.PDF, and *.jpg. Please refer to the pixl™ Real-Time PCR Platform user manual for further instructions[12].

Bio Molecular Systems Mic Instrument

Results are available to view and download in .pdf format by navigating to the "Results" tab, choosing the appropriate experiment, and selecting the "Export" option on the Mic PCR software. Results are also available to download in the following formats: *.csv, *.PDF, *.html, *.mht, *.rtf, *.docx, *.xlsx, *.xls, *.txt, and *.image. Please refer to the Bio Molecular Systems Mic Instrument user manual for further instructions[13].

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.

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.

Table 2. Interpretation of patient sample results[16].

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≤40	+	Ct≤36	+/-	Ct≤30	Report as **POSITIVE**
SARS-CoV-2 **NEGATIVE**	-	Not Detected	+	Ct≤36	+	Ct≤30	Report as **NEGATIVE**
Indeterminant	+/-	Ct 0-40	- / Ct≥36	-/Ct≥36	+	Ct≤30	Repeat Test*
Indeterminant	+/-	Ct 0-40	+	Ct≤36	- / Ct≥30	- / Ct≥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.

LIMITATIONS OF THE PROCEDURE

- This device is not designed as the sole means of diagnosis of infectious disease. By the inherent nature of the technology used for nucleic acid extraction and detection, nucleic acid can be detected from dead organisms. The Intended Use is limited to the detection of the presence of the nucleic acid signature of an organism, and not the diagnosis of disease or disease state.
- 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 instructions for use and the real-time PCR platforms (ie. Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well)[9], Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well[9], Applied Biosystems™ 7500 Fast Dx[10] Real-Time PCR Instrument, Bio-Rad CFX96 Touch™/CFX384 Touch™[11] Real-Time PCR Detection System, pixl™ Real-Time PCR Platform[12], Bio Molecular Systems Mic Instrument[13]) are necessary to avoid erroneous results.
- Good laboratory technique is essential for the proper performance of this assay. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of all materials and reagents.
- 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.
- As with all PCR-based *in vitro* diagnostic tests, extremely low levels of target below the limit of detection of the assay may be detected, but such results may not be reproducible.
- False negative results may occur due to loss of nucleic acid from inadequate collection, transport or storage of specimens, or due to inadequate cell lysis and/or extraction. The RNase P and IAC targets have been added to the test to aid in the identification of properly collected specimens, specimens that contain inhibitors to PCR amplification, and as a control for reagent integrity and of the assay system as a whole.
- The BioGX *Xfree*™ COVID-19 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.

- 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.
- The BioGX *Xfree*™ COVID-19 requires the use of three (3) optical channels on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well), Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well[9], Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™/CFX384 Touch™ Real-Time PCR Detection System, pixl™ Real-Time PCR Platform, Bio Molecular Systems Mic Instrument.

PERFORMANCE CHARACTERISTICS

Analytical Sensitivity - Direct Patient Sample

Limit of Detection (LoD) was determined by serially diluting quantified inactivated SARS-CoV-2 (USA-WA1/2020; Zeptometrix catalog number NATSARS(CoV2)-ST) into negative NP swabs samples in Copan UTM, Copan ESwab, VTM and saline transport media. The LoD confirmation for each collection device (i.e. Copan UTM, Copan ESwab, VTM or saline) were confirmed by reliably detecting the number of inactivated SARS-CoV-2 viruses/mL in ≥95% of the samples tested. LoD confirmation was performed using the following real-time PCR platforms: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well), Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well, Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™/CFX384 Touch™ Real-Time PCR Detection System, pixl™ Real-Time PCR Platform, Bio Molecular Systems Mic Instrument. 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. REF: 500-003-XMP LoD confirmation - **direct addition** of contrived samples using SARS-CoV-2 USA-WA1/2020.

Instrument	Transport Media	Concentration [Copies/ mL]	N1-gene		RNase P	IAC
			Agreement with Expected Result (% Positive)	Mean Ct	Mean Ct	Mean Ct
Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well)	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
Applied Biosystems™ QuantStudio™ 5 384-well	UTM/VTM	2,000	20/20 (100%)	34.6	31.8	23.1
	Saline	2,500	19/20 (95%)	34.8	28.1	19.0
	ESwab	3,000	20/20 (100%)	32.9	31.9	16.3
Applied Biosystems™ 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
Bio-Rad 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
Bio-Rad 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
pixl™ Real-Time PCR Platform	UTM/VTM	2000	19/20 (95%)	31.1	31.9	25.6
	Saline	3000	19/20 (95%)	32.1	29.3	25.2
Bio Molecular Systems Mic	UTM/VTM	1000	21/22 (96%)	34.0	29.2	26.8
	Saline	1500	22/22 (100%)	33.6	31.2	26.4
	ESwab	2000	22/22 (100%)	33.9	29.1	27.3

Analytical Sensitivity - Extracted Patient Sample

Limit of Detection (LoD) was determined by serially diluting quantified inactivated SARS-CoV-2 (USA-WA1/2020; Zeptometrix catalog number NATSARS(CoV2)-ST) into negative NP swabs samples in the respective transport media types (i.e. Copan ESwab, Copan UTM, saline or VTM) extracted with Qiagen Viral RNA Mini Kit. The LoD confirmation for each collection device was confirmed by reliably detecting the number of inactivated SARS-CoV-2 viruses/mL in ≥95% of the samples tested. LoD confirmation was performed using the following real-time PCR platforms: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well), Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 384-well, Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™/CFX384 Touch™ Real-Time PCR Detection System, pixl™ Real-Time PCR Platform, Bio Molecular Systems Mic Instrument. The LoD for the assay using Extracted Patient Samples on the validated platforms ranged from 125 - 750 copies/mL (**Table 4**).

Table 4. REF: 500-003-XMP LoD confirmation - **extracted*** contrived samples using SARS-CoV-2 USA-WA1/2020.

Instrument	Transport Media	Concentration [Copies/ mL]	N1-gene		RNase P	IAC
			Agreement with Expected Result (% Positive)	Mean Ct	Mean Ct	Mean Ct
Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well)	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
Applied Biosystems™ QuantStudio™ 5 384-well	UTM/VTM	250	19/20 (95%)	32.0	26.4	15.8
	Saline	250	19/20 (95%)	34.8	27.8	17.4
	ESwab	500	19/20 (95%)	34.7	27.9	23.9
Applied Biosystems™ 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
Bio-Rad 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
Bio-Rad CFX384 Touch™	UTM/VTM	500	20/20 (100%)	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
pixl™ Real-Time PCR Platform	UTM/VTM	250	20/20 (100%)	33.3	29.7	24.8
	Saline	500	20/20 (100%)	32.5	27.6	25.0
Bio Molecular Systems Mic	UTM/VTM	750	22/22 (100%)	33.6	27.3	25.3
	Saline	750	22/22 (100%)	33.6	29.3	25.2
	ESwab	500	22/22 (100%)	33.5	28.6	26.2

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

Analytical Sensitivity - Pooled Direct Patient Sample

Limit of Detection (LoD) was determined by serially diluting quantified inactivated SARS-CoV-2 (USA-WA1/2020; Zeptometrix catalog number NATSARS(CoV2)-ST) into negative NP swabs samples collected in the respective transport media types (i.e. Copan ESwab, Copan UTM, saline or VTM). To create contrived 5 patient pools for each transport media, four individual negative NP swab samples were pooled and combined with a fifth sample that was spiked with a defined concentration of quantified SARS-CoV-2 virus. The LoD confirmation for pooled samples in each respective collection device was confirmed by reliably detecting the number of inactivated SARS-CoV-2 viruses/mL in ≥95% of the samples tested. LoD confirmation was performed using the following real-time PCR platforms: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2mL 96-well), Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument, Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and pixl™ Real-Time PCR Platform. The LoD for the assay using Pooled Direct Patient Samples on the validated platforms ranged from 2,000 - 6,000 copies/mL (**Table 5**).

Table 5. REF: 500-003-XMP LoD confirmation - **pooled** contrived samples using SARS-CoV-2 USA-WA1/2020.

Instrument	Transport Media	Concentration [Copies/ mL]	N1-gene		RNase P	IAC
			Agreement with Expected Result (% Positive)	Mean Ct	Mean Ct	Mean Ct
Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well)	UTM/VTM	4,000	20/20 (100%)	34.55	30.19	23.19
	Saline	3,000	19/20 (95%)	34.11	29.42	20.86
	ESwab	4,000	20/20 (100%)	34.03	30.65	23.70
Applied Biosystems™ 7500 Fast Dx	UTM/VTM	5,000	20/20 (100%)	36.84	33.68	28.78
	Saline	2,000	20/20 (100%)	36.07	31.86	25.87
	ESwab	4,000	20/20 (100%)	35.81	33.05	28.29
Bio-Rad CFX96 Touch™	UTM/VTM	5,000	20/20 (100%)	37.22	31.24	27.07
	Saline	3,000	19/20 (95%)	37.10	31.07	26.05
	ESwab	6,000	20/20 (100%)	34.80	35.47	30.65
pixl™ Real-Time PCR Platform	UTM/VTM	4,000	19/20 (95%)	33.50	31.40	25.60
	Saline	5,000	19/20 (95%)	33.30	30.30	25.20

Inclusivity (*in-silico*)

The BioGX SARS-CoV-2 N1 primers and probes are identical in sequence to those reported in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. The CDC reported an *in-silico* analysis of primer and probe sequences within their IFU (CDC-006-00019, Rev 05)[3]. An alignment was performed with the oligonucleotide primer and probe sequences of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic Panel with all publicly available nucleic acid sequences for 2019-nCoV in GenBank as of June 20, 2020, to demonstrate the predicted inclusivity of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic panel. All the alignments show >99% identity of the CDC panel to the available 2019-nCoV sequences with the exception of one sequence with a mismatch frequency of >1% in the N1 probe, all other sequences assessed had mismatch frequencies <1%. From the mismatch frequencies with <1%, two separate deposited sequences were observed to have two mismatches in the N1 probe and N1 reverse primer.

SARS-CoV-2 Alpha variants [B.1.1.7 lineage, Qx]: To confirm the test's ability to detect the SARS-CoV-2 Alpha variants first observed circulating in the United Kingdom, BioGX ran *in-silico* inclusivity analysis against the Alpha variant sequences present in the GISAID database as of March 31, 2022. The available SARS-CoV-2 Alpha variant genomes demonstrated >99% homology to the N gene region and are expected to be detected by the assay. The *in-silico* analysis shows the analyzed SARS-CoV-2 Alpha variants do not possess mutations within the targeted region of the N gene that are expected to affect detection of SARS-CoV-2 by the BioGX *Xfree*™ COVID-19 Direct RT-PCR.

SARS-CoV-2 Beta variants [B.1.351, B.1.351.2, B.1.351.3 lineages]: To confirm the BioGX test's ability to detect the SARS-CoV-2 Beta variants, first observed circulating in South Africa, BioGX ran *in-silico* inclusivity analysis against the Beta variant sequences present in the GISAID database as of March 31, 2022. The available SARS-CoV-2 Beta variant genomes demonstrated >99% homology to the N gene region and are expected to be detected by the assay. The *in-silico* analysis of the currently circulating strains of the SARS-CoV-2 Beta variants do not possess mutations within the the targeted region of the N gene that are expected to affect detection of SARS-CoV-2 by the BioGX *Xfree*™ COVID-19 Direct RT-PCR.

SARS-CoV-2 Gamma variants [P.1 and P.1.x lineages]: To confirm the BioGX test's ability to detect the SARS-CoV-2 Gamma variants, first observed circulating in Brazil, BioGX ran *in-silico* inclusivity analysis against the Gamma variant sequences present in the GISAID database as of March 31, 2022. The available SARS-CoV-2 Gamma variant genomes demonstrated >99% homology to the N gene region are expected to be detected by the assay. The *in-silico* analysis of the currently circulating strains of the SARS-CoV-2 Gamma variant do not possess mutations within the the targeted region of the N gene that are expected to affect detection of SARS-CoV-2 by the BioGX *Xfree*™ COVID-19 Direct RT-PCR.

SARS-CoV-2 Delta variants [B.1.617.2 and AY.x lineages]: To confirm the BioGX test's ability to detect the SARS-CoV-2 Delta variants, first observed circulating in India, BioGX ran *in-silico* inclusivity analysis against the Delta variant sequences present in the GISAID database as of March 31, 2022. The available SARS-CoV-2 Delta variant genomes demonstrated >99% homology to the N gene region and are expected to be detected by the assay. The *in-silico* analysis of the currently circulating strains of the SARS-CoV-2 Delta variant do not possess mutations within the the targeted region of the N gene that are expected to affect detection of SARS-CoV-2 by the BioGX *Xfree*™ COVID-19 Direct RT-PCR.

SARS-CoV-2 Omicron variants [B.1.529 and BAx lineages]: To confirm the BioGX test's ability to detect the SARS-CoV-2 Omicron variants, first observed circulating in South Africa, BioGX ran *in-silico* inclusivity analysis against the Omicron variant sequences present in the GISAID database as of March 31, 2022. The available SARS-CoV-2 Omicron variant genomes demonstrated >99% homology to the N gene region and are expected to be detected by the assay. The *in-silico* analysis of the currently circulating strains of the SARS-CoV-2 Omicron variant do not possess mutations within the the targeted region of the N gene that are expected to affect detection of SARS-CoV-2 by the BioGX *Xfree*™ COVID-19 Direct RT-PCR.

Cross-Reactivity (Analytical Specificity)

Select high priority organisms represented in a respiratory specimen were evaluated using the oligonucleotide primers and probes in BioGX *Xfree*™ COVID-19 by *in-silico* BLASTn alignments with an expected threshold of 5000 for potential cross-reactivity (**Table 6**). The combination of primers and probes did not produce significant sequence homology or identity which would result in cross-reactivity reporting to any of the selected high priority respiratory specimens. The primers and probes were further evaluated against high priority pathogens within the same genetic family (e.g. human coronavirus strains, MERS-coronavirus). The resultant *in-silico* analysis showed no significant homology to pathogens from the same genetic family (excluding SARS-CoV-2).

Microbial Interference

None of the high priority microbial species above (also refer to **Table 6**), which could potentially be present in the clinical specimens, showed any *in-silico* homology to the primers and probes used in the assay, and the BioGX SARS-CoV-2 N1 primers and probes are identical in sequence to those reported in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, therefore a microbial interference study specifically against these strains is not required per US FDA recommendations.[3,16] Additionally, during the development and validation of the assay, SARS-CoV-2 negative NP swabs obtained in kind, were collected from individuals presenting with illness, de-identified of patient information, and tested with the BioGX *Xfree*™ COVID-19. These patient samples resulted in 0% false positive detection. Additionally, in the event a sufficient microbial contamination were to occur due to an improperly collected specimen, the IAC would indicate if the excessive microbial load would inhibit RNA reverse transcription, amplification, and detection.

Table 6. *Xfree*™ COVID-19 Direct RT-PCR primers and probes *in-silico* analysis of high priority organisms.

Organism	SARS-CoV-2 (N1) Percent Identity	Organism	SARS-CoV-2 (N1) Percent Identity
Human coronavirus 229E	0%	*Chlamydia pneumoniae*	0%
Human coronavirus OC43	0%	*Haemophilus influenzae*	0%
Human coronavirus HKU1	0%	*Legionella pneumophila*	0%
Human coronavirus NL63	0%	*Mycobacterium tuberculosis*	0%
MERS-coronavirus	0%	*Streptococcus pneumoniae*	0%
SARS-coronavirus	0%	*Streptococcus pyogenes*	0%
Adenovirus	0%	*Bordetella pertussis*	0%
Human Metapneumovirus (hMPV)	0%	*Mycoplasma pneumoniae*	0%
Parainfluenza virus 1-4	0%	*Pneumocystis jirovecii* (PJP)	0%
Influenza A & B	0%	*Candida albicans*	0%
Enterovirus (e.g. EV68)	0%	*Pseudomonas aeruginosa*	0%
Respiratory syncytial virus	0%	*Staphylococcus epidermidis*	0%
Rhinovirus	0%	*Streptococcus salivarius*	0%

Interfering Substances

Potential interfering substances were spiked into pooled Copan UTM negative nasopharyngeal swab samples containing SARS-CoV-2 (USA-WA1/2020; Zeptometrix catalog number NATSARS(CoV2)-ST) at 5X LoD (5,000 copies/mL). **Table 7** summarizes the replicate runs (n=3) conducted for each substance with the indicated concentrations. All runs were conducted with direct sample addition to the master mix and analyzed with both the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System and Bio-Rad CFX96 Touch™ Real-Time PCR Detection System.

The BioGX *Xfree*™ COVID-19 assay utilizes a single stranded RNA Internal Amplification Control (IAC) included in every reaction. The single stranded RNA IAC shows conditions for amplification within the reaction were suitable for reverse transcription, PCR amplification, and generation of fluorescence to take place. In the event a Direct tested sample contains interfering substances which are detrimental to the real time PCR, by either inhibiting reverse transcription or cDNA polymerization, the IAC would fail to amplify and report. The results interpretation of such a sample as an indeterminate, requires the analyst to first retest the sample, and if it is still inhibitory to collect another sample for testing. A similar strategy is employed for extracted samples. As such, false negative results due to inhibition are mitigated by the incorporation of the single stranded RNA IAC into each test to identify inhibition, and providing a reflexive testing strategy that indicates need for new sample collection or re-testing with established extraction procedure.

Table 7. Analysis of interfering substances.

Potential Interfering substance	Final concentration (in sample)	Agreement with expected results			
		Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well)		Bio-Rad CFX96 Touch™	
		Positive NP	Negative NP	Positive NP	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%

Potential Interfering substance	Final concentration (in sample)	Agreement with expected results			
		Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well)		Bio-Rad CFX96 Touch™	
		Positive NP	Negative NP	Positive NP	Negative NP
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%
Tobacco	0.5% v/v	100%	100%	100%	100%
Human DNA	10 ng/µL	100%	100%	100%	100%
White blood cells	10% v/v ($4.5x10^5$-$1.10x10^6$/mL)	100%	100%	100%	100%
Whole Blood	2.5% v/v	100%	100%	100%	100%
Whole Blood	1.0% v/v	100%	100%	100%	100%

Clinical Performance

Clinical performance of the BioGX *Xfree*™ COVID-19 assay (REF: 500-003-XMP) was evaluated using clinical samples previously identified and confirmed with the US FDA-EUA comparator method: Linea™ COVID-19 Assay Kit. Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) was determined using 60 individual positive and 35 individual negative clinical samples, collected by qualified healthcare professionals. 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 sample analysis on the Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well) from high, medium, and low positive clinical patient samples (n=60) and negative clinical patient samples (n=35), Direct sample addition achieved a 95.45% PPA and 100% NPA when compared to the comparator method (**Table 8**).

Testing with Extracted Patient Samples

The clinical samples were extracted with Qiagen Viral RNA Mini Kit following the manufacturer's recommendations where 140 µL of clinical sample is extracted and then eluted with 50 µL RNase-free water. The eluted nucleic acid template (5 µL) is added to 10 µL of the BioGX *Xfree*™ master mix. Extracted high, medium, and low positive clinical patient samples (n=60) and negative clinical patient samples (n=35) yielded PPA and NPA of 100% and 94.29%, respectively, when analyzed on the Applied Biosystems™ QuantStudio™ 5 (0.2 mL 96-well) (**Table 9**).

Table 8. REF: 500-003-XMP Clinical performance percent agreement of Direct sample addition with Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well) and comparator method (Linea™ COVID-19 assay).

QuantStudio™ 5 (0.2 mL 96-well) Direct Sample Addition	Comparator Method: **Linea™ COVID-19 assay**	
Xfree™ COVID-19 Direct RT-PCR	*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% (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 9. REF: 500-003-XMP Clinical performance percent agreement of Extracted sample addition with Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well) and comparator method (Linea™ COVID-19 assay).

QuantStudio™ 5 (0.2 mL 96-well) Qiagen Extracted Nucleic Acid	Comparator Method: **Linea™ COVID-19 assay**	
Xfree™ COVID-19 Direct RT-PCR	*Positive*	*Negative*
Positive	60	2*
Negative	0	33
Positive Percent Agreement (95%CI)	100% (94.0-100.0%)	
Negative Percent Agreement (95%CI)	94.3% (81.4-98.4%)	

*The two samples that tested positive with the BioGX *Xfree*™ COVID-19 assay (REF: 500-003-XMP) and negative with the comparator method were tested with a third, highly sensitive RT-PCR US FDA-EUA authorized test. Both samples tested positive with the second RT-PCR US FDA-EUA authorized test.

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APPENDIX A

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well) - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5 Real-Time PCR System** 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 Real-Time PCR 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)	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	1	1	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 "Target 1" 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 Real-Time PCR System (0.2 mL 96-well) - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5 Real-Time PCR System** 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 Real-Time PCR 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 A2**):
        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).

Table A2. QuantStudio™ 5 thermocycling summary - Extracted 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)	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	1	1	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 “Target 1” 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”.

Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (0.2 mL 96-well) - Thermocycling Programing

Pooling - Direct Pooled Patient Sample (40 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5 Real-Time PCR System** 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 500003XMPDirectPooledQS5Rev01.edt
    b. Instrument Type: QuantStudio™ 5 Real-Time PCR 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 **40.0 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 A3**):
        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 A3. QuantStudio™ 5 thermocycling summary - Pooled 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)	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	1	1	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 "Target 1" 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 ‘500003XMPDirectPooledQS5Rev01’ 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™ QuantStudio™ 5 Real-Time PCR System (384-well) - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5 Real-Time PCR System (384-well)** 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:
    1) Name: User defined or 500003XMPDirectQS5_384Rev01.edt
    2) Instrument Type: QuantStudio™ 5, 384-well Real-Time PCR System
    3) Block Type: 384-Well 0.2 mL
    4) Experiment type: Standard Curve
    5) Chemistry: TaqMan Reagents
    6) 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 B1**):
        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 B1. QuantStudio™ 5 (384-well) 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)	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	1	1	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 "Target 1" 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 ‘500003XMPDirectQS5_384Rev01.edt’ 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 Real-Time PCR System (384-well) - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **QuantStudio™ 5 Real-Time PCR System (384-well)** 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 500003XMPExtractedQS5_384Rev01.edt
   b. Instrument Type: QuantStudio™ 5 Real-Time PCR System , 384-well System
   c. Block Type: 384-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 B2**):
      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).

Table B2. QuantStudio™ 5 (384-well) 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)	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	1	1	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 “Target 1” 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 ‘500003XMPExtractedQS5_384Rev01.edt’ 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 C

Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument** 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 C1**):
    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 C1. 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)	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	1	1	40		

Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument- Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument** 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 C2**):
    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 C2. Applied Biosystems™ 7500 thermocycling summary - Extracted 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)	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	1	1	40		

Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument - Thermocycling Programing

Pooled Direct Patient Sample (40 µL reaction volume)

Please refer to the following steps for **Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument** 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 **40.0 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 C3**):
    i. Hold Stage: Step 1
        1. Auto ramp rate
        2. 46°C
        3. 20 minutes
    ii. Hold Stage: Step 2
        4. Auto ramp rate
        5. 95°C
        6. 5 minutes
    iii. PCR Stage: Step 1
        7. Auto ramp rate
        8. 95°C
        9. 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: 40
        2. Run Mode: Fast 7500
        3. Data Calculation: Stage 3, step 2

e. Select the "Analysis" tab and click "Analysis settings".

vii. 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

viii. 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

ix. 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”.

g. Select File -> Save. Set your desired name and your template is saved.

Table C3. Applied Biosystems™ 7500 thermocycling summary - Pooled 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)	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	1	1	40		

APPENDIX D

Bio-Rad CFX96 Touch™ Real-Time PCR Detection System - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **Bio-Rad CFX96 Touch™ Real-Time PCR Detection System** 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 '500003XMPDirectCFX96v2'
iv. Save as type 'Protocol File (*.prcl)'
v. Select Save
vi. Click Ok in the protocol editor window

Table D1. 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)	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	1	1	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
   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

Bio-Rad CFX96 Touch™ Real-Time PCR Detection System - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **Bio-Rad CFX96 Touch™ Real-Time PCR Detection System** 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 D2**):
    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 ‘500003XMPExtractCFX96v2’
        iv. Save as type ‘Protocol File (*.prcl)’

v. Select Save
vi. Click Ok in the protocol editor window

Table D2. CFX96 Touch™ thermocycling summary - Extracted Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	**Step 1**	**Step 2**	**Step 1**	**Step 2**	**Step 3**
Temperature (°C)	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	1	1	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
   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.

Bio-Rad CFX96 Touch™ Real-Time PCR Detection System - Thermocycling Programing

Pooling - Direct Pooled Patient Sample (40 µL reaction volume)

Please refer to the following steps for **Bio-Rad CFX96 Touch™ Real-Time PCR Detection System** 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 **40 µL**
    b. Click “Insert Step” to add a total of five total steps and program as follows (**Table D3**):
    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 ‘500003XMPDirectPooledCFX96Rev01’
        iv. Save as type ‘Protocol File (*.prcl)’

v. Select Save
vi. Click Ok in the protocol editor window

Table D3. CFX96 Touch™ thermocycling summary - Pooled Direct Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	**Step 1**	**Step 2**	**Step 1**	**Step 2**	**Step 3**
Temperature (°C)	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	1	1	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
   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 ‘500003XMPDirectPooledCFX96PlateRev01’
      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

APPENDIX E

Bio-Rad CFX384 Touch™ Real-Time PCR Detection System - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **Bio-Rad CFX384 Touch™ Real-Time PCR Detection System** 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 E1**):
    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 E1. 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)	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	1	1	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
   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 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.
   e. 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
   f. 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

Bio-Rad CFX384 Touch™ Real-Time PCR Detection System - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for **Bio-Rad CFX384 Touch™ Real-Time PCR Detection System** 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 E2**):
    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

Table E2. CFX384 Touch™ thermocycling summary - Extracted Patient Sample.

PCR Cycle Summary					
	Hold Stage		PCR Stage		
Conditions	**Step 1**	**Step 2**	**Step 1**	**Step 2**	**Step 3**
Temperature (°C)	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	1	1	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
   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 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

APPENDIX F

pixl™ Real-Time PCR Platform - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for pixl™ Real-Time PCR Platform touchpad manual programming specific for Direct patient sample testing.

1. Open the "BioGX qPCR" pixl™ Real-Time PCR Platform software
2. Select "Test" on the left side home screen
3. Within the "Test" screen, tap "New Experiment" to proceed to the Sample Setup screen
4. In the Experiment Name section, enter the test name.
    a. Experiment Name: User-defined or 500003XMPDirExtpixlRev01
5. In the Channel section, select the dye and define the target identified in each channel.
    a. Select Channel 1
        i. From the drop-down menu select: FAM
        ii. Name the Channel: RNaseP
    b. Select Channel 3
        i. From the drop-down menu select: ROX
        ii. Name the Channel: CoV2-N1
    c. Select Channel 4
        i. From the drop-down menu select: Cy5
        ii. Name the Channel: IAC
    d. Integration Time for pixl™ Real-Time PCR Platform optimized tests are recommended to remain set to: auto.
    e. To name the wells by sample name, select the appropriate well and next to "Name" rename well to whatever is desired for the experiment.
    f. If necessary, a barcode scanner can be plugged into the USB port located on the front panel of the instrument to facilitate sample ID capture.
6. Tap Next to go to the Cycling Program screen
7. Add, remove, or edit a stage or step.
8. Adjust the temperature and time of a stage of step, number of cycles, and data capture step.
    a. Ensure Auto Integration Time is selected
    b. Within the section of the thermocycling parameters, an additional step will need to be added by selecting the "+" next to "Const-tempe Stage".
    c. Starting at the first "Pre-Denat" stage enter the following parameters (**Table F1**)

i. Pre-Denat: Step 1
    1. 46°C
    2. 600 seconds
ii. Pre-Denat2: Step 1
    1. 95°C
    2. 150 seconds
iii. Cycle Period: Step 1
    1. 95°C
    2. 5 seconds
iv. Cycle Period: Step 2
    1. 59°C
    2. 12 seconds
v. Hold: Step 1
    1. 50°C
    2. 60 seconds

d. Save the template file before starting the run by clicking “Save Template” in the upper right corner.

Table F1: pixl™ Real-Time PCR Platform thermocycling summary: Direct Patient Samples.

PCR Cycle Summary					
	Pre-Denat Stage		Cycle Period Stage		Hold Stage
Conditions	Step 1	Step 2	Step 1	Step 2	Step 1
Temperature (°C)	46	95	95	59	50
Duration (sec)	600	150	5	12	60
Image Step	No	No	No	Yes	No
No. of PCR Cycles	1	1	40		1

9. Start Run
    a. Select Start in the bottom right corner of the touchpad, this will automatically take you to the “Run” tab
10. After run completion, navigate to the “Results” tab and select the appropriate test to set the threshold for each channel used
    a. Set Ct Low Limit to 13
    b. Set Ct Threshold (%) to 6.0
11. Save File (Report tab)
    a. In the upper right corner, select “Export” to save .json file, name file as desired or “500003XMPDirExtpixlRev01”
    b. Insert a USB key in the USB port located on the front panel of the instrument to save the results file
    c. Select “CSV” to save the file as an excel document
    d. Select “PDF” to save the file as a PDF document
12. Select “JSON” to save the file as a JSON document

pixl™ Real-Time PCR Platform - Thermocycling Programing

Extracted Patient Sample (15 µL reaction volume)

Please refer to the following steps for pixl™ Real-Time PCR Platform touchpad manual programming specific for Extracted patient sample testing.

1. Open the "BioGX qPCR" pixl™ Real-Time PCR Platform app
2. Select "Test" on the left side home screen
3. Within the "Test" screen, tap "New Experiment" to proceed to the Sample Setup screen
4. In the Experiment Name section, enter the test name.
    a. Experiment Name: User-defined or 500003XMPDirExtpixlRev01
5. In the Channel section, select the dye and define the target identified in each channel.
    a. Select Channel 1
        i. From the drop-down menu select: FAM
        ii. Name the Channel: RNaseP
    b. Select Channel 3
        i. From the drop-down menu select: ROX
        ii. Name the Channel: CoV2-N1
    c. Select Channel 4
        i. From the drop-down menu select: Cy5
        ii. Name the Channel: IAC
    d. Integration Time for pixl™ Real-Time PCR Platform optimized tests are recommended to remain set to: auto.
    e. To name the wells by sample name, select the appropriate well and next to "Name" rename well to whatever is desired for the experiment.
    f. If necessary, a barcode scanner can be plugged into the USB port located on the front panel of the instrument to facilitate sample ID capture.
6. Tap Next to go to the Cycling Program screen
7. Add, remove, or edit a stage or step.
8. Adjust the temperature and time of a stage of step, number of cycles, and data capture step.
    a. Ensure Auto Integration Time is selected
    b. Within the section of the thermocycling parameters, an additional step will need to be added by selecting the "+" next to "Const-tempe Stage".
    c. Starting at the first "Pre-Denat" stage enter the following parameters (**Table F2**)
        i. Pre-Denat: Step 1
            1. 46°C

2. 600 seconds

ii. Pre-Denat2: Step 1
1. 95°C
2. 150 seconds

iii. Cycle Period: Step 1
1. 95°C
2. 5 seconds

iv. Cycle Period: Step 2
1. 59°C
2. 12 seconds

v. Hold: Step 1
1. 50°C
2. 60 seconds

d. Save the template file before starting the run by clicking “Save Template” in the upper right corner.

Table F2: pixl™ Real-Time PCR Platform thermocycling summary: Extracted Patient Samples.

PCR Cycle Summary					
	Pre-Denat Stage		Cycle Period Stage		Hold Stage
Conditions	Step 1	Step 2	Step 1	Step 2	Step 1
Temperature (°C)	46	95	95	59	50
Duration (sec)	600	150	5	12	60
Image Step	No	No	No	Yes	No
No. of PCR Cycles	1	1	40		1

9. Start Run
    a. Select Start in the bottom right corner of the touchpad, this will automatically take you to the “Run” tab
10. After run completion, navigate to the “Results” tab and select the appropriate test to set the threshold for each channel used
    a. Set Ct Low Limit to 13
    b. Set Ct Threshold(%) to 6.0

11. Save Results File (Report tab)
    a. In the upper right corner, select “Export” to save .json file, name file as desired or “500003XMPDirExtpixlRev01”
    b. Insert a USB key in the USB port located on the front panel of the instrument to save the results file
    c. Select “CSV” to save the file as an excel document
    d. Select “PDF” to save the file as a PDF document
    e. Select “JSON” to save the file as a JSON document

pixl™ Real-Time PCR Platform - Thermocycling Programing

Direct Pooled Patient Sample (40 µL reaction volume)

Please refer to the following steps for pixl™ Real-Time PCR Platform touchpad manual programming specific for Direct Pooled patient sample testing.

1. Open the "BioGX qPCR" pixl™ Real-Time PCR Platform app
2. Select "Test" on the left side home screen
3. Within the "Test" screen, tap "New Experiment" to proceed to the Sample Setup screen
4. In the Experiment Name section, enter the test name.
    a. Experiment Name: User-defined or 500003XMPDirExtpixlRev01
5. In the Channel section, select the dye and define the target identified in each channel.
    a. Select Channel 1
        i. From the drop-down menu select: FAM
        ii. Name the Channel: RNaseP
    b. Select Channel 3
        i. From the drop-down menu select: ROX
        ii. Name the Channel: CoV2-N1
    c. Select Channel 4
        i. From the drop-down menu select: Cy5
        ii. Name the Channel: IAC
    d. Integration Time for pixl™ Real-Time PCR Platform optimized tests are recommended to remain set to: auto.
    e. To name the wells by sample name, select the appropriate well and next to "Name" rename well to whatever is desired for the experiment.
    f. If necessary, a barcode scanner can be plugged into the USB port located on the front panel of the instrument to facilitate sample ID capture.
6. Tap Next to go to the Cycling Program screen
7. Add, remove, or edit a stage or step.
8. Adjust the temperature and time of a stage of step, number of cycles, and data capture step.
    a. Ensure Auto Integration Time is selected
    b. Within the section of the thermocycling parameters, an additional step will need to be added by selecting the "+" next to "Const-tempe Stage".
    c. Starting at the first "Pre-Denat" stage enter the following parameters (**Table F3**)
        i. Pre-Denat: Step 1
            1. 46°C

2. 600 seconds

ii. Pre-Denat2: Step 1
1. 95°C
2. 150 seconds

iii. Cycle Period: Step 1
1. 95°C
2. 5 seconds

iv. Cycle Period: Step 2
1. 59°C
2. 12 seconds

v. Hold: Step 1
1. 50°C
2. 60 seconds

d. Save the template file before starting the run by clicking “Save Template” in the upper right corner.

Table F3: pixl™ Real-Time PCR Platform thermocycling summary: Pooled Direct Patient Samples.

PCR Cycle Summary					
	Pre-Denat Stage		Cycle Period Stage		Hold Stage
Conditions	Step 1	Step 2	Step 1	Step 2	Step 1
Temperature (°C)	46	95	95	59	50
Duration (sec)	600	150	5	12	60
Image Step	No	No	No	Yes	No
No. of PCR Cycles	1	1	40		1

9. Start Run
    a. Select Start in the bottom right corner of the touchpad, this will automatically take you to the “Run” tab
10. After run completion, navigate to the “Results” tab and select the appropriate test to set the threshold for each channel used
    a. Set Ct Low Limit to 13
    b. Set Ct Threshold(%) to 6.0

11. Save Results File (Report tab)
    a. In the upper right corner, select “Export” to save .json file, name file as desired or “500003XMPDirExtpixlRev01”
    b. Insert a USB key in the USB port located on the front panel of the instrument to save the results file
    c. Select “CSV” to save the file as an excel document
    d. Select “PDF” to save the file as a PDF document
    e. Select “JSON” to save the file as a JSON document

APPENDIX G

Bio Molecular Systems Mic Instrument - Thermocycling Programing

Direct Patient Sample (20 µL reaction volume)

Please refer to the following steps for **Bio Molecular Systems Mic Instrument** manual programming specific for Direct Patient sample testing.

1. Launch the Mic PCR software.
2. In the top left of the software, select “New”
    a. Select “Assay”
3. Select the “Information” tab on the banner on the left side of the software.
4. Under “Chemistry type” select “Hydrolysis Probes”
5. In the “Targets” section:
    a. Enter “RNase P” as the first target and select FAM as the Reporter Dye
    b. Enter “N1” as the second target and select “Cal Fluor 610” as the Reporter Dye.
    c. Enter “IAC” as the third target and select “Q670” as the Reporter Dye.
6. The remaining fields in the “Information” tab do not need to be populated.
7. Select “Profile”
    a. On the top right of the software banner, select “Fast Taq” for the Temperature Control.
    b. Set the volume to **20 µL** for direct sample addition testing.
8. Thermocycling programming parameters (**Table G1**)
    a. Pre-Denat: Step 1
        i. 46°C
        ii. 1200 seconds
    b. Pre-Denat2: Step 1
        i. 95°C
        ii. 300 seconds
    c. Cycle Period: Step 1
        i. 95°C
        ii. 3 seconds
    d. Cycle Period: Step 2
        i. 62°C
        ii. 20 seconds

iii. Select the camera to turn on acquisition.

e. Cycle Period: Step 3

i. 70°C

ii. 5 seconds

f. Set the number of cycles to 40.

9. In the top left of the software select “Save As”

a. Name the file as desired. The file extension should be “.micassay”

Table G1. Bio Molecular Systems Mic Instrument thermocycling summary: Direct Patient Samples.

PCR Cycle Summary					
	Pre-Denat Stage		Cycle Period Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Temperature (°C)	46	95	95	62	70
Duration (sec)	1200	300	3	20	5
Image Step	No	No	No	Yes	No
No. of PCR Cycles	1	1	40		

Bio Molecular Systems Mic Instrument - Thermocycling Programing

Direct Patient Sample (15 µL reaction volume)

Please refer to the following steps for **Bio Molecular Systems Mic Instrument** manual programming specific for Direct Patient sample testing.

1. Launch the Mic PCR software.
2. In the top left of the software, select "New"
    a. Select "Assay"
3. Select the "Information" tab on the banner on the left side of the software.
4. Under "Chemistry type" select "Hydrolysis Probes"
5. In the "Targets" section:
    a. Enter "RNase P" as the first target and select FAM as the Reporter Dye
    b. Enter "N1" as the second target and select "Cal Fluor 610" as the Reporter Dye.
    c. Enter "IAC" as the third target and select "Q670" as the Reporter Dye.
6. The remaining fields in the "Information" tab do not need to be populated.
7. Select "Profile" tab located on the software banner.
    a. On the top right of the software banner, select "Fast Taq" for the Temperature Control.
    b. Set the volume to **15 µL** for direct sample addition testing.
8. Thermocycling programming parameters (**Table G2**)
    a. Pre-Denat: Step 1
        i. 46°C
        ii. 1200 seconds
    b. Pre-Denat2: Step 1
        i. 95°C
        ii. 300 seconds
    c. Cycle Period: Step 1
        i. 95°C
        ii. 3 seconds
    d. Cycle Period: Step 2
        i. 62°C
        ii. 20 seconds
        iii. Select the camera to turn on acquisition.
    e. Cycle Period: Step 3
        i. 70°C

ii. 5 seconds

f. Set number of cycles to 40.

9. In the top left of the software select “Save As”

b. Name the file as desired. The file extension should be “.micassay”

Table G2. Bio Molecular Systems Mic Instrument thermocycling summary: Extracted Patient Samples.

PCR Cycle Summary					
	Pre-Denat Stage		Cycle Period Stage		
Conditions	Step 1	Step 2	Step 1	Step 2	Step 3
Temperature (°C)	46	95	95	62	70
Duration (sec)	1200	300	3	20	5
Image Step	No	No	No	Yes	No
No. of PCR Cycles	1	1	40		

REVISION HISTORY

Revision	Date	Description of Change
09	07APR2023	Update of real-time PCR platform names and typographical edits.
08	13JAN2023	Update name "BioGX pixl.16" to "pixl™ Real-Time PCR Platform"
07	22SEP2022	Update Table 1A and 1B regarding N1 gene and IAC Expected Ct values
06	19AUG2022	Update of BioGX pixl.16 run file names to 500003XMPDirExtpixlRev01 and remove all references regarding US FDA-EUA product approval and use of product in the United States.
05	20MAY2022	Update branding, Update with addition of BioGX pixl™ Real-Time PCR platform for detection of direct addition of individual samples, detection of nucleic acid extracted individual samples and detection of direct addition of pooled patient samples, Update with addition of Bio Molecular Systems Mic Instrument for detection of direct addition of individual samples and detection of nucleic acid extracted individual samples. Update with addition of Applied Biosystems™ Quantstudio™ 5 (384-well) platform for detection of direct addition of individual samples and detection of nucleic acid extracted individual samples. Update with addition of BioGX pixl™ Real-Time PCR platform and consumables part numbers, Update with addition of Bio Molecular Systems Mic Instrument consumable part numbers, Update with addition of Applied Biosystems™ Quantstudio™ 5 (384-well) platform consumable part numbers, Update temperature range of shipping condition, Update to eliminate storage of rehydrated master mix, Update use of BioGX External Control for Direct or Extracted Sample Testing for BioGX pixl™ Real-Time PCR platform, Update Table 2, Update in-silico analysis section, Update Tables 7 and 8, Correction to CalRed610 settings in Appendix E, Addition of Appendix F, Addition of Appendix G
4.1	11OCT2021	Update Intended Use Section to indicate performance of test was not evaluated with all circulating variants.
04	01APR2021	Update number of reactions (tests) per tube to number of reactions (tests) per kit. Update Table 2 to simplify interpretation, Update to add direct analysis of pooled patient sample, validated real-time PCR instruments compatible with sample types, number of tests per sample type, pooled sample set up, pooled LoD summary, Appendix A and Appendix C.
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.
02	18 NOV 2020	Update equipment and materials required but not provided section, Update specimen preparation section, update positive control and patient result interpretation, update limit of detection table, update clinical performance, update reference section
01	09 OCT 2020	Initial Release

SYMBOLS

Symbol	Meaning	Symbol	Meaning
REF	Catalog number	Σ	Contains sufficient for <n> tests
CE	CE mark of conformity	IVD	*In vitro* diagnostic medical device
	Do not reuse		Temperature limitation
LOT	Batch code		Keep dry
	Caution		Keep away from sunlight
	Consult instructions for use		Expiration date
	Manufacturer		Biological Risks
CONTROL	Control	EC REP	Authorized Representative

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