



BillionToOne, Inc.
1035 O'Brien Drive
Menlo Park, CA 94025 USA
www.billiontoone.com

Instructions For Use: qSanger-COVID-19 Assay

VERSION 2.0

FOR EMERGENCY USE AUTHORIZATION (EUA) ONLY

FOR IN VITRO DIAGNOSTIC USE

FOR PRESCRIPTION USE ONLY (Rx ONLY)

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PART 1. INTENDED USE

The qSanger-COVID-19 Assay is a Sanger sequencing-based, RNA extraction-free diagnostic test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory swab specimens (such as nasal swab, mid-turbinate swab, nasopharyngeal swab, and oropharyngeal swab specimens) from individuals suspected of COVID-19 by their healthcare provider. Testing is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, and meet requirements to perform high complexity tests.

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

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

The qSanger-COVID-19 Assay is intended for use by qualified clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR, Sanger Sequencing and *in vitro* diagnostic procedures. The qSanger-COVID-19 Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

PART 2. PRODUCT DESCRIPTION

The qSanger-COVID-19 Assay is a Sanger sequencing-based test for detection of SARS-CoV-2 RNA. The SARS-CoV-2 sequences and a spike-in sequence serving as an internal control are amplified with a primer pair designed to detect RNA from SARS-CoV-2 in upper respiratory swab specimens collected from patients who are suspected of COVID-19. Instruments employed to perform the test from sample collection to result include a thermal cycler (e.g. Applied Biosystems Veriti Thermal Cycler) and Sanger sequencing instrument (e.g. Applied Biosystems 3730x1 DNA Analyzer).

PART 3. Reagents, Materials, and Storage

3.1. Materials Provided

Component (for 2000 reactions, with 20% overage)	Storage
12 mL Reagent A1: SARS-CoV-2 Primer Mix with Spike-In Reagent	-20°C (-15 to -25°C)
3 mL Enzyme A2: Luna® WarmStart® RT Enzyme Mix (20x)	
30 mL Enzyme A3: Luna® Universal Probe One Step Reaction Mix No ROX (2x)	
5 mL Reagent B1: Sanger Sequence Primer	
0.2 mL Reagent C1: SARS-CoV-2 Primer Mix without Spike-In Reagent	

The shelf life of this kit is 1 year based on the LUNA enzyme expiration period. Shelf life is subject to change as stability studies are ongoing.

3.2.a. Materials Required But Not Provided - Reagents

Item	Vendor (Manufacturer)	Catalog Number
REAGENTS		
Nuclease Free Water	MLS*	NA
ExoSAP-IT PCR Product Cleanup Reagent	ThermoFisher Scientific (Affymetrix)	78201.1.ML
BigDye Terminator v3.1 Cycle Sequencing Kit	Fisher Scientific (Applied Biosystems)	43-374-55
Agencourt CleanSeq	Beckman Coulter	A29151
Absolute Ethanol	MLS*	NA
Hi-Di Formamide	Thermo Fisher Scientific (Applied Biosystems)	4401457
3730 Running Buffer (10X)	Thermo Fisher Scientific (Applied Biosystems)	4335613
POP-7 Polymer for 3730/3730xl DNA Analyzers	Thermo Fisher Scientific (Applied Biosystems)	4363929

REAGENTS USED AS CONTROLS		
Ultrapure RNase-Free Water	MLS*	NA
SeraCare AccuPlex™ SARS-CoV-2 Reference Material Kit	SeraCare	0505-0126
REAGENTS / CONSUMABLES EXCLUSIVELY USED IN REAGENT PREP		
50 mL Conical Tube	MLS*	NA
5 mL Conical Tubes	MLS*	NA

*MLS- any major laboratory supplier/ fisherscientific.com

3.2.b. Materials Required but Not Provided - Consumables

Item	Vendor (Manufacturer)	Catalog Number
Low Retention Pipette Tips	MLS*	NA
0.2 mL PCR Tube Strips	MLS*	NA
15 mL Conical Tubes	MLS*	NA
Reagent Reservoir	MLS*	NA
Adhesive PCR Plate Seal	Thermo Scientific	AB0558
Eppendorf twin.tec 96 Well LoBind PCR Plate, Semi-skirted	Eppendorf	E0030129504
MicroAmp Optical 96-Well Reaction Plate	ThermoFisher Scientific (Applied Biosystems)	4306737
Plate Septa, 96-Well	ThermoFisher Scientific (Applied Biosystems)	4315933
3730xl Capillary Array, 36 cm	ThermoFisher Scientific (Applied Biosystems)	4331244

*MLS- any major laboratory supplier/ fisherscientific.com

3.2.c. Materials Required but Not Provided - Equipment

Equipment	Vendor (Manufacturer)	Catalog Number
Veriti Thermal Cycler	ThermoFisher Scientific (Applied Biosystems)	4375786
Vortexer	MLS*	NA
Mini Centrifuge	MLS*	NA
Centrifuge	MLS*	NA
DynaMag-96 Side Magnet	ThermoFisher Scientific (Invitrogen)	12331D
3730xl DNA Analyzer	ThermoFisher Scientific (Applied Biosystems)	3730XL
Single Channel Manual Pipettes	MLS*	NA
Multichannel Manual Pipette	MLS*	NA

*MLS- any major laboratory supplier/ fisherscientific.com

PART 4. WARNING AND PRECAUTIONS

4.1. General Warnings and Precautions

- For *in vitro* diagnostic use
- For prescription use only
- For use under FDA emergency use authorization (EUA) only
- The qSanger-COVID-19 Assay has not been FDA cleared or approved;
- The qSanger-COVID-19 Assay has been authorized by FDA under an Emergency Use Authorization (EUA) for use by laboratories, which are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, and meet the requirements to perform high complexity tests;
- The qSanger-COVID-19 Assay has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.
- The qSanger-COVID-19 Assay is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.

- Only personnel proficient in handling infectious materials and trained in the use of the qSanger-COVID-19 Assay should perform this procedure.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.
- Laboratory personnel must be familiar with the protocol and all instrumentation used.
- RNA is highly unstable and extra precaution should be employed to ensure it does not degrade in samples.
- Maintain separate areas for sample accessioning, RT-PCR and the set-up of the sequencing reaction to minimize the risk of contamination with amplifiable material in the sample processing steps.
- Maintain dedicated equipment (e.g., pipettes, microcentrifuges, vortexes) and supplies (e.g., 1.5mL tubes, pipette tips, gloves) for assay reagent set-up and handling of extracted nucleic acids.
- Workflow must always be in the direction of clean area to dirty area.
- Carefully clean pipettes, containers, and surfaces with RNase Away, RNaseZap, or a similar product before and between sample batches. Change gloves frequently.
- Never bring extracted nucleic acid or amplification products into the assay set-up area.
- Use nuclease-free, aerosol barrier (filter) pipette tips only.
- Do not use reagents past their expiration date.

4.2. Biohazard

All patient samples should be handled as if infectious, using good laboratory procedures as outlined in Biosafety in Microbiological and Biomedical Laboratories and in the CLSI Document M29-A4. Samples should be processed in a properly ventilated biosafety cabinet. Use appropriate personal protective equipment. Sanitize all surfaces before and after using 70% alcohol or 10% bleach.

4.3. Chemical Hazards

Consult MSDS for hazards relating to specific reagents. Follow all recommended precautions, including those for handling and disposal.

PART 5. QUALITY CONTROLS

Positive and negative test controls must be included to accurately interpret patient test results.

Control	Controls For
No-RNA negative control	Contamination with genomic SARS-CoV-2 sequences
Positive Control: AccuPlex SARS-CoV-2 Positive Reference Material	Positive signal measurement (correct reverse transcription and amplification)
No Template - No Spike-in Control	Contamination with either spike-in or genomic SARS-CoV-2 sequences

These controls should be prepared as three separate reactions in addition to up to 93 unknown specimen reactions in a 96-well plate. Please see Section 7.1, Steps 6 and 7.

PART 6. SAMPLE COLLECTION, HANDLING AND STORAGE

Sample collection device is not a part of the assay kit. Patient samples must be collected according to appropriate laboratory guidelines. All testing for COVID-19 should be conducted in consultation with a healthcare provider. We recommend using CDC guidelines for sample collection of upper respiratory swab specimens and sample storage (<https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html>).

Specimen should be processed within 48 hours from collection and stored at 2-25°C during that time as per the manufacturer's instructions. If they cannot be tested within 48 hours samples should be stored frozen at -70°C or colder.

Upper respiratory swab Collection

Once the swabs have been collected as per the CDC guidelines above, it is recommended to use Universal Transport Medium (UTM) System for transportation and storage of swabs.

PART 7. WORK INSTRUCTIONS

The *qSanger-COVID-19* Assay does not require RNA extraction for normal assay performance. VTM from NP/OP swabs can be added directly to the reactions.

7.1. Amplification

1. Obtain and label a PCR plate for PCR Amplification.
2. Carefully clean the workspace with RNase Away.
3. Remove reagents from -20°C storage and allow to thaw on ice.

Briefly vortex the *Reagent A1 (Primer and Spike-in Mix)* tube and centrifuge to collect liquid. Return to ice for reaction assembly. Do not vortex the Luna reagents. Invert *Enzyme A3 (Luna® Universal Probe One-Step Reaction Mix)* and *Enzyme A2 (NEB Luna® RT Enzyme)* tubes to mix. Briefly spin down the tube and centrifuge to collect liquid. Return to ice for reaction assembly.

- In an RNase-free conical tube, combine the following reagents at the listed volumes to prepare the *Assembled Reaction Master Mix*. Invert the tube to mix well and briefly centrifuge to collect liquid.

Note: calculated volumes account for 10% excess for pipetting error.

<i>Assembled Reaction Master Mix</i>		
Reagent	Volume (µL) For N unknown samples	Volume (µL) For full 96-well plate (N = 93)
Enzyme A3 (Luna® Universal Probe One-Step Reaction Mix)	= 12.5*(N+3)*1.1	1320
Reagent A1 (Primer and Spike-in Mix)	= 5*(N+3)*1.1	528
Enzyme A2 (NEB Luna® RT Enzyme)	= 1.25*(N+3)*1.1	132
Total	= 18.75*(N+3)*1.1	1980

- Add 18.75 µL Assembled Master Mix to each well to be tested.
Tip: Use a reagent trough and a multichannel pipette to fill the PCR Amplification Plate.
- Add 6.25 µL of control sample to each appropriate well, as detailed below. Gently pipette up and down to mix.
A01: Positive Control
B01: RNase-free water
- Add 12.5 µL of Enzyme A3, 5 µL of Reagent C1, 1.25 µL of Enzyme A2, and 6.25 µL of RNase-free water to the well C01 for the no-template, no-spike-in control.
- Add 6.25 µL *unknown sample* to each remaining well. Gently pipette up and down to mix.
- Carefully apply plate seal to the PCR Amplification Plate such that it is airtight. Press each well to make sure it is sealed.
- Briefly spin down the PCR Amplification Plate using the short spin feature on a plate centrifuge.
- Load the PCR Amplification Plate on the thermal cycler and start the PCR Amplification Program.
- Add the following PCR Amplification program to the thermal cycler. Ensure a heated lid is used.

PCR Amplification Program		
Temperature	Time (mm:ss)	Cycles
55°C	20:00	1x
95°C	01:00	
95°C	00:20	40x
55°C	01:00	
60°C	01:00	
60°C	05:00	
4°C	∞	1x

7.2. PCR Clean-up, using ExoSAP

Prepare Reagents and Instrument

1. Obtain and label a PCR plate for PCR Clean-up.
2. Obtain reagents from -20°C storage. Flick and invert tube to mix. Briefly spin down to collect liquid.
3. Obtain the PCR Amplification plate containing PCR Amplification products. Ensure reaction wells are well mixed. Briefly spin down with plate centrifuge to collect liquid.
4. Add the following PCR Clean-up program to the thermal cycler. Ensure a heated lid is used.

PCR Clean-up Program		
Temperature	Time (mm:ss)	Cycles
37°C	15:00	1x
80°C	15:00	
4°C	∞	

Assemble and Run PCR-Clean Up Reaction

1. In a labeled 1.5 mL microcentrifuge tube, combine the following reagents at the listed volumes to prepare the *ExoSAP Master Mix*. Invert the tube to mix well and briefly centrifuge to collect liquid.

Note: calculated volumes account for 10% excess for pipetting error.

<i>ExoSAP Master Mix (Full 96-well Plate)</i>		
Reagent	Volume (µL) For n PCR Amplified samples	Volume (µL) For full 96-well plate (n = 96)
<i>Nuclease-free Water</i>	= 3*n*1.1	316.8
<i>ExoSAP-IT™ PCR Product Cleanup Reagent</i>	= 2*n*1.1	211.2
Total	= 5*n	528

2. Add 5 µL of *ExoSAP Master Mix* to each appropriate well.
Tip: Use a reagent trough and a multichannel pipette to fill the PCR Clean-up Plate.
3. Add 2 µL of PCR Amplification product to each appropriate well.
Tip: Use a multichannel pipette to fill the PCR Clean-up Plate
4. Carefully apply plate seal to the PCR Clean-up Plate such that it is airtight. Press each well to make sure it is sealed.
5. Briefly spin down the PCR Clean-up Plate using the short spin feature on a plate centrifuge.
6. Load the PCR Clean-up Plate on the thermal cycler and start the PCR Clean-up Program.

7.3. Cycle Sequencing, using BigDye v3.1 Kit

Prepare Reagents and Instrument for Sequencing Reaction

1. Obtain and label a PCR plate for Cycle Sequencing.
2. Obtain reagents from -20°C storage. Flick and invert tube to mix. Briefly spin down to collect liquid.
3. Obtain PCR Clean-up Plate containing PCR Clean-up products. Ensure reaction wells are well mixed. Briefly spin down with plate centrifuge to collect liquid.
4. Add the following Cycle Sequencing program to the thermal cycler. Ensure a heated lid is used.

Important note: Adjust the ramp rate such that it is $\leq 1^\circ\text{C}/\text{sec}$ during cycling steps.

Cycle Sequencing Program			
Temperature	Time (mm:ss)	Ramp Rate*	Cycles
96°C	1:00	100%*	1x
96°C	0:10	37%*	30x
50°C	0:05	42%*	

60°C	4:00	37%*	
60°C	4:00	100%*	1x
4°C	∞	100%*	

* Ramp rate setting used for BillionToOne validation runs on the Veriti Thermal Cycler.

Assemble and Run Sequencing Reaction

1. In a labeled 1.5 mL microcentrifuge tube, combine the following reagents at the listed volumes to prepare the *Cycle Sequencing Master Mix*. Invert the tube to mix well and briefly centrifuge to collect liquid.

Note: calculated volumes account for 10% excess for pipetting error.

<i>Cycle Sequencing Master Mix (Full 96-well Plate)</i>		
Reagent	Volume (μL) For n cycle sequenced samples	Volume (μL) For full 96-well plate (n = 96)
<i>Nuclease-free Water</i>	$= 2 * n * 1.1$	211.2
<i>Reagent B1 (Sanger Sequencing Primer)</i>	$= 1 * n * 1.1$	105.6
<i>BigDye Ready Reaction Mix</i>	$= 2 * n * 1.1$	211.2
Total	$= 5 * n * 1.1$	3960

2. Add 5 μL of *Cycle Sequencing Master Mix* to each appropriate well.
Tip: Use a reagent trough and a multichannel pipette to fill the Cycle Sequencing Plate.
3. Add 5 μL of PCR Clean-up Product from PCR Clean-up Plate to each appropriate well.
Tip: Use a multichannel pipette to fill the Cycle Sequencing Plate.
4. Carefully apply plate seal to the Cycle Sequencing Plate such that it is airtight. Press each well to make sure it is sealed.
5. Spin down Cycle Sequencing Plate using the short spin feature on a plate centrifuge.
6. Load the Cycle Sequencing Plate in the thermal cycler and start the Cycle Sequencing program.

7.4. Dye-Terminator Clean-Up, Using CleanSEQ Kit

Prepare Reagents and Instrument for the Dye Terminator Clean-Up

1. Obtain and label a PCR plate for Sanger Sequencing.

- Obtain *Hi-Di Formamide* from -20°C storage. Allow to thaw at room temperature. Briefly spin down to collect liquid.
- Obtain *CleanSEQ Beads* from 4°C storage. Vortex the bottle until the beads are well mixed and in suspension.
- Obtain Cycle Sequencing Plate containing Cycle Sequencing products. Ensure reaction wells are well mixed. Briefly spin down with plate centrifuge to collect liquid. Add “+ Clean-up” to the plate label.
- Prepare Sanger Sequencing Plate by adding $10\ \mu\text{L}$ of *Hi-Di Formamide* to sample wells. Add $20\ \mu\text{L}$ of nuclease-free water to any remaining wells in the plate.
Tip: Preparation of the Sanger Sequencing Plate can be done while waiting for elution incubations to complete.

Assemble and Run Dye-Terminator Clean-Up reaction.

- In a conical tube, combine V_{Water} mL of water and V_{Ethanol} mL of absolute ethanol to create *85% Ethanol*. Vortex to mix and briefly centrifuge to collect liquid.

<i>85% Ethanol*</i>	
Reagent	Volume (mL)
<i>Nuclease-free Water</i>	4.2
<i>Absolute Ethanol</i>	23.8
Total	28.0

* Note: the above quantity of *85% Ethanol* is sufficient for 1 full 96-well plate. The *85% Ethanol* should be made fresh for each reaction and used within 24 hrs of creation.

- Pipette $10\ \mu\text{L}$ of *CleanSeq Beads* to each reaction well in the Cycle Sequencing Plate.
- Pipette $42\ \mu\text{L}$ of *85% Ethanol* into each well. Pipette up and down until well mixed.
- Position Cycle Sequencing + Clean-up Plate on the magnetic bead plate. Incubate at room temperature for 3 minutes or until the solution is clear.

Important Note: Maintain Cycle Sequencing + Clean-up Plate on the magnetic bead plate for subsequent steps.

- Remove supernatant using a pipette.
Tip: Use a multichannel pipette to remove the supernatant.
- Perform Wash 1:
 - Add $100\ \mu\text{L}$ of *85% Ethanol* to each well. Incubate for 30 seconds.
Tip: Use a multichannel pipette to add 85% Ethanol to each well.
 - Remove supernatant using a pipette.
Tip: Use a multichannel pipette to remove the supernatant from each well.
- Perform Wash 2:
 - Add $100\ \mu\text{L}$ of *85% Ethanol* to each well. Incubate for 30 seconds.

Tip: Use a multichannel pipette to add 85% Ethanol to each well.

2. Remove supernatant using a pipette.

Tip: Use a multichannel pipette to remove the supernatant from each well.

8. Incubate at room temperature for 10 minutes or until dry.

Tip: Prepare the Sanger Sequencing Plate while waiting for elution incubations to complete.

9. Add 40 μ L of nuclease free water to the sample. Pipet up and down to mix. Incubate at room temperature for 5 minutes to elute the sample.

Tip: Prepare the Sanger Sequencing Plate while waiting for elution incubations to complete.

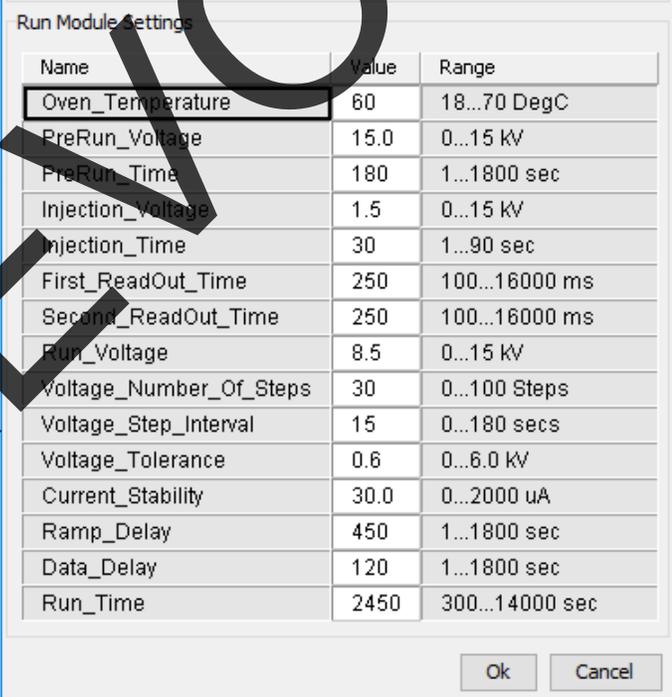
10. Transfer 10 μ L of eluted samples to the appropriate wells in the Sanger Sequencing plate.

11. Carefully apply plate seals to the Cycle Sequencing + Clean-up and Sanger Sequencing plates such that they are airtight. Press each well to make sure it is sealed.

7.5. Capillary Electrophoresis

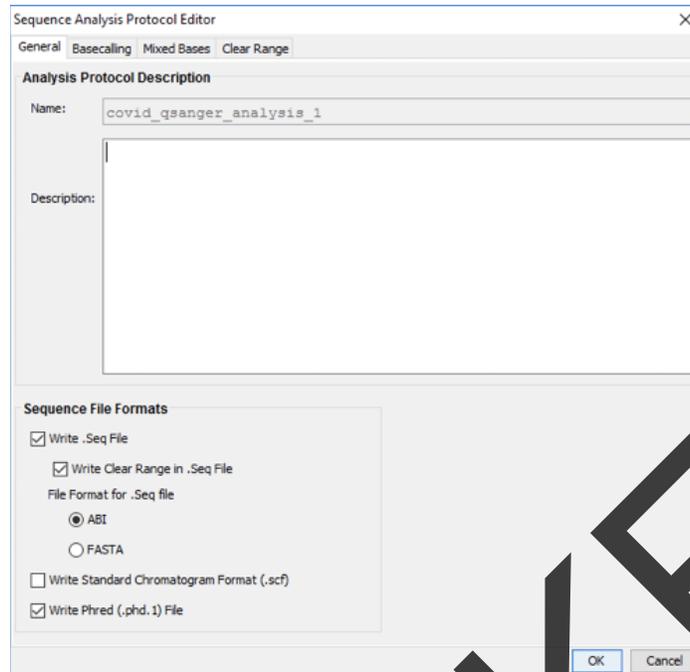
Set-up

1. Add COVID-19 qSanger run module. Open the 3730xl Data Collection Software (or similar software on any ABI instrument). Navigate to the Module Manager and open the Run Module Editor. Add the following run module.



Name	Value	Range
Oven_Temperature	60	18...70 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.5	0...15 kV
Injection_Time	30	1...90 sec
First_ReadOut_Time	250	100...16000 ms
Second_ReadOut_Time	250	100...16000 ms
Run_Voltage	8.5	0...15 kV
Voltage_Number_Of_Steps	30	0...100 Steps
Voltage_Step_Interval	15	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	450	1...1800 sec
Data_Delay	120	1...1800 sec
Run_Time	2450	300...14000 sec

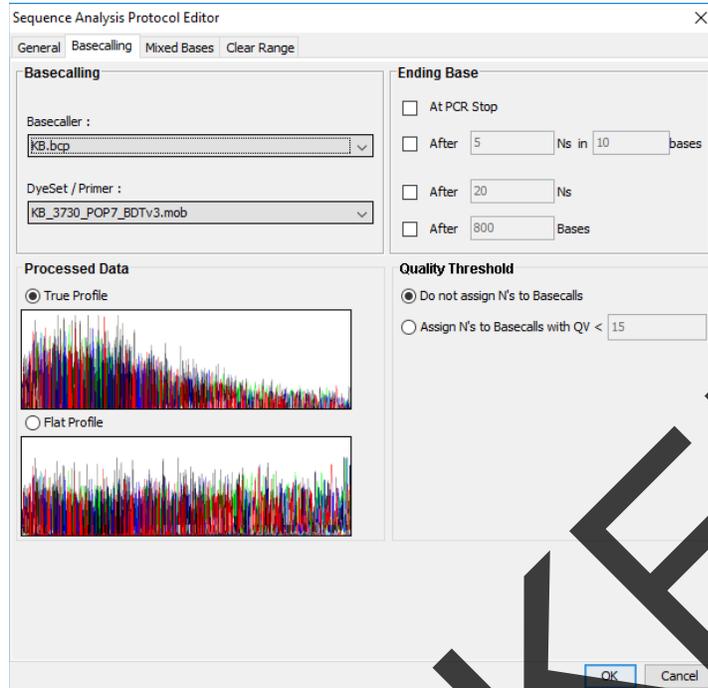
2. Add COVID-19 qSanger analysis settings. Navigate to the Protocol Manager and open the Sequence Analysis Protocol Editor.
 - a. Under the tab titled “General”, ensure that the following settings are selected.



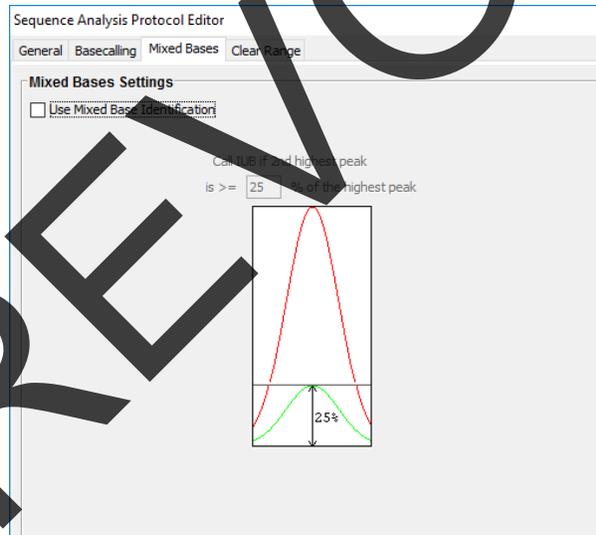
- b. Under the tab titled “Basecalling”, ensure that the following settings are selected.
Important Note: Ensure that all “Ending Base” settings are toggled off.

Note: In particular, “PCR stop” function should be set to off in the ThermoFisher Data Collection Software in order to obtain the 3’ viral sequence chromatogram. Without turning this off, base calling usually stops prematurely and misses bases calls for weakly positive samples.

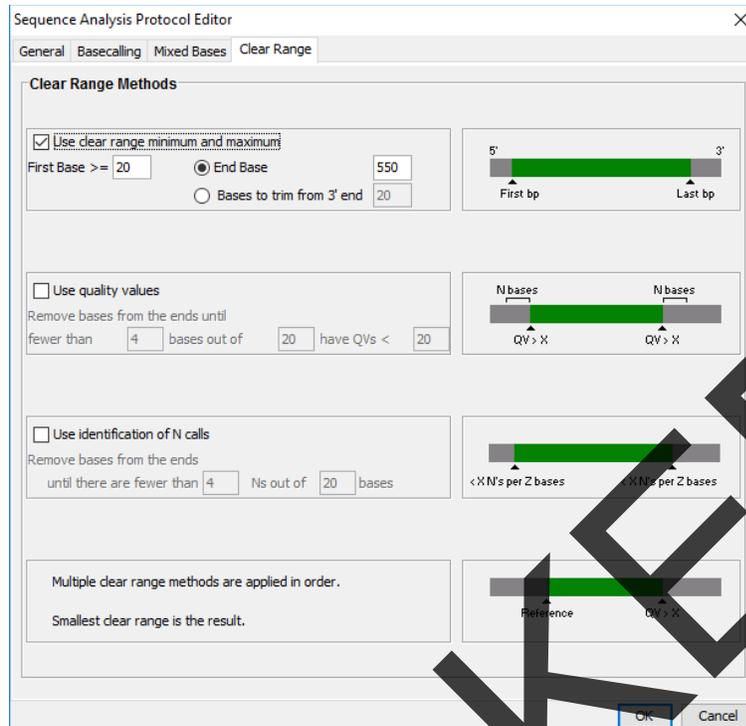
However, this also means that the software will continue call bases even in the absence of clear signal. The automatic base-calls of the sequence shown at the top of the chromatogram should not be used for any purpose. A trained personnel should inspect the chromatogram for the presence and absence of spike-in and viral sequences, as shown in later steps.



- c. Under the tab titled “Mixed Bases”, ensure that the following settings are selected.



- d. Under the tab titled “Clear Range”, ensure that the following settings are selected.



3. For each run, create a new Sequencing Analysis Plate Record.
 - a. Open the 3730xl Data Collection Software. Navigate to the Plate Manager.
 - b. Create a New Plate then complete the Sequencing Analysis Plate record by inputting the appropriate number of samples and selecting the COVID-19 Instrument and Analysis Protocols.
 - c. Add desired plates to the Run Scheduler.
4. Ensure that the DNA Analyzer is ready for a run. Complete all required maintenance activities prior to loading.
5. Consult the user manual for general instrument maintenance, set-up, and related troubleshooting instructions. ([link](#))

Protocol

1. Obtain Sanger Sequencing Plate containing Cycle Sequencing products that have undergone Dye-Terminator Clean-up. Centrifuge at 1000 g for 1 minute in a plate centrifuge. Ensure that no large bubbles are present in any of the wells.
2. Remove plate seal from Sanger Sequencing Plate and replace with a plate septa. Prepare 3730xl plate assembly by placing the septa-capped plate into a plate retainer.
3. Load the plate assembly onto the 3730xl instrument and begin the scheduled run.

PART 8. DATA ANALYSIS

8.1. Assessment of qSanger Results

Open ab1 files and inspect for the presence and absence of spike-in and native SARS-CoV-2 RNA sequences. See data interpretation section for more details.

DATA ANALYSIS SOFTWARE	MANUFACTURER	DESCRIPTION
3730xl Data Collection Software 5 and Sequencing Analysis Software 7	ThermoFisher Scientific	Automated Chromatogram Processing Software
(Optional) BillionToOne qSanger-COVID-19 QC Software	BillionToOne, Inc.	QC metrics to facilitate clinical personnel analysis of chromatograms and identification of 'no-sequence' repeat test samples

Raw data collected by the 3730xl instrument is automatically processed using the above software for manual inspection. Processed ab1 files should include chromatograms clear of noise and show base calls for the entire length of the amplicon.

Below, please see examples of types of traces for controls:

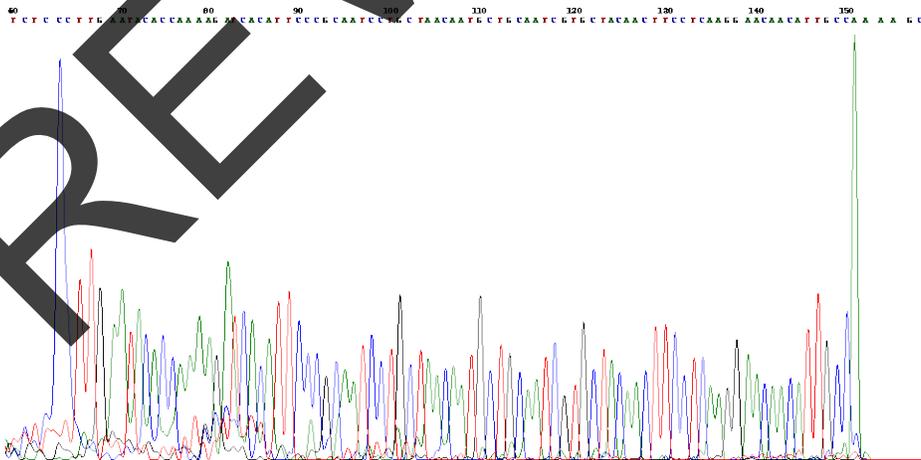


Figure 1 AccuPlex SARS-CoV-2 Negative Reference Material (only spike-in sequence should be present)

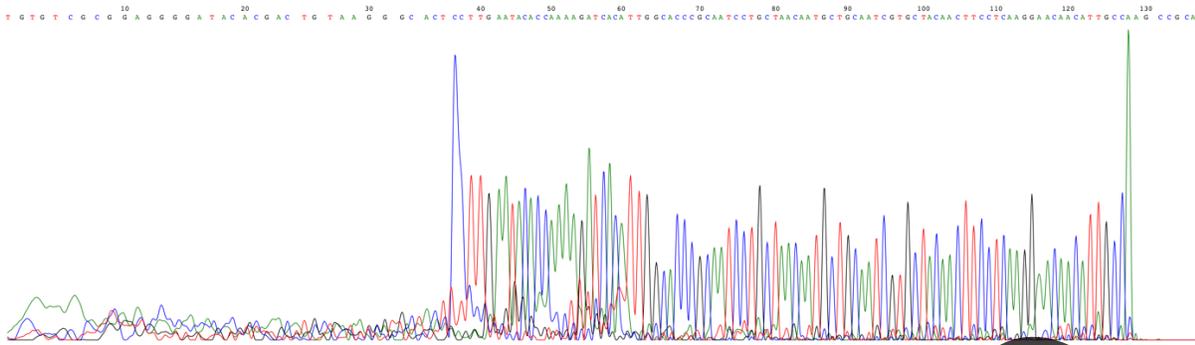


Figure 4 – Example of a strongly positive result

SARS-CoV-2 (COVID-19) RNA detected (weakly positive):

Samples with moderate or relatively low concentrations of viral RNA result in electropherograms where both the endogenous and spike-in sequence are visible. The example shown in Figure 5 had relatively low RNA input, resulting in spike-in signal that is greater than viral sequence signal. The signal from the viral sequence, which is a longer genomic product, is visible at the 3' end as well as in the mixed signal for the overlapping sequence. Note that the **“PCR stop” setting on the sequencing instrument must be turned off** in order to obtain this data. Please note that because the PCR stop is turned off, the base-caller may continue to make calls even if the sequence completely ends; the base-calls at 3' end that are indicated at the top of the chromatogram should not be used for any purposes. Instead, the chromatogram itself should be inspected for the repeat sequence that would indicate the presence of viral SARS-CoV-2 RNA.

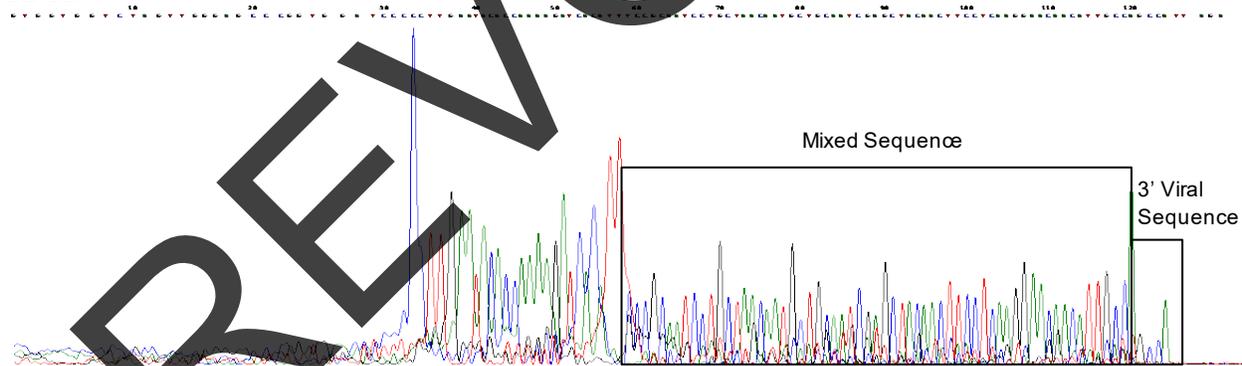


Figure 5 – Example of a weakly positive result. Note the mixed sequence highlighted above as well as the 3' viral sequence. Note that the 3' viral sequence alone is sufficient to indicate the presence of viral genomic sequence.

Negative Results

In negative samples, signal is produced only by the spike-in sequence.

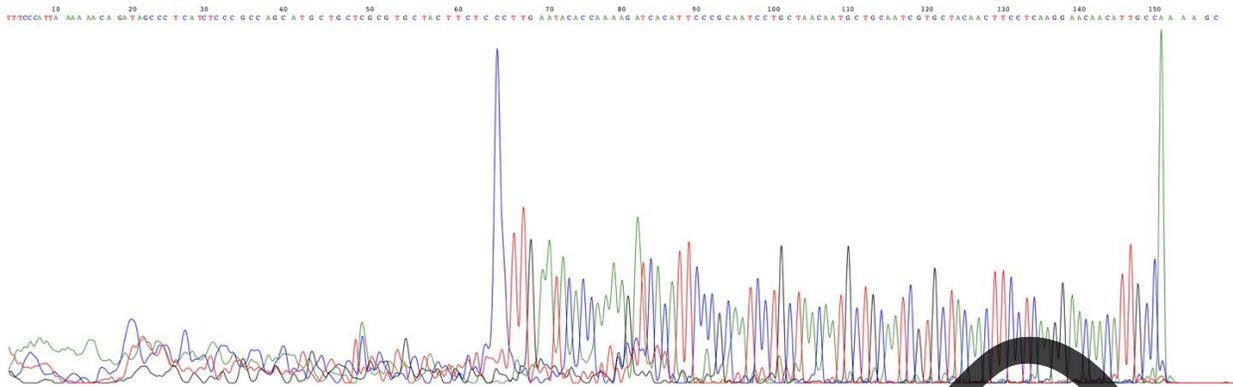


Figure 6 – Example of a negative result. The sequence TTCCC at 88 bp is the site of the deletion (see Figure 7 below).

Negative samples show unmixed sequence matching the SARS-CoV-2 genome, differing by a 4 bp deletion. A comparison of highly positive (top image) vs. negative (spike-in only, lower image) electropherograms can be seen below.

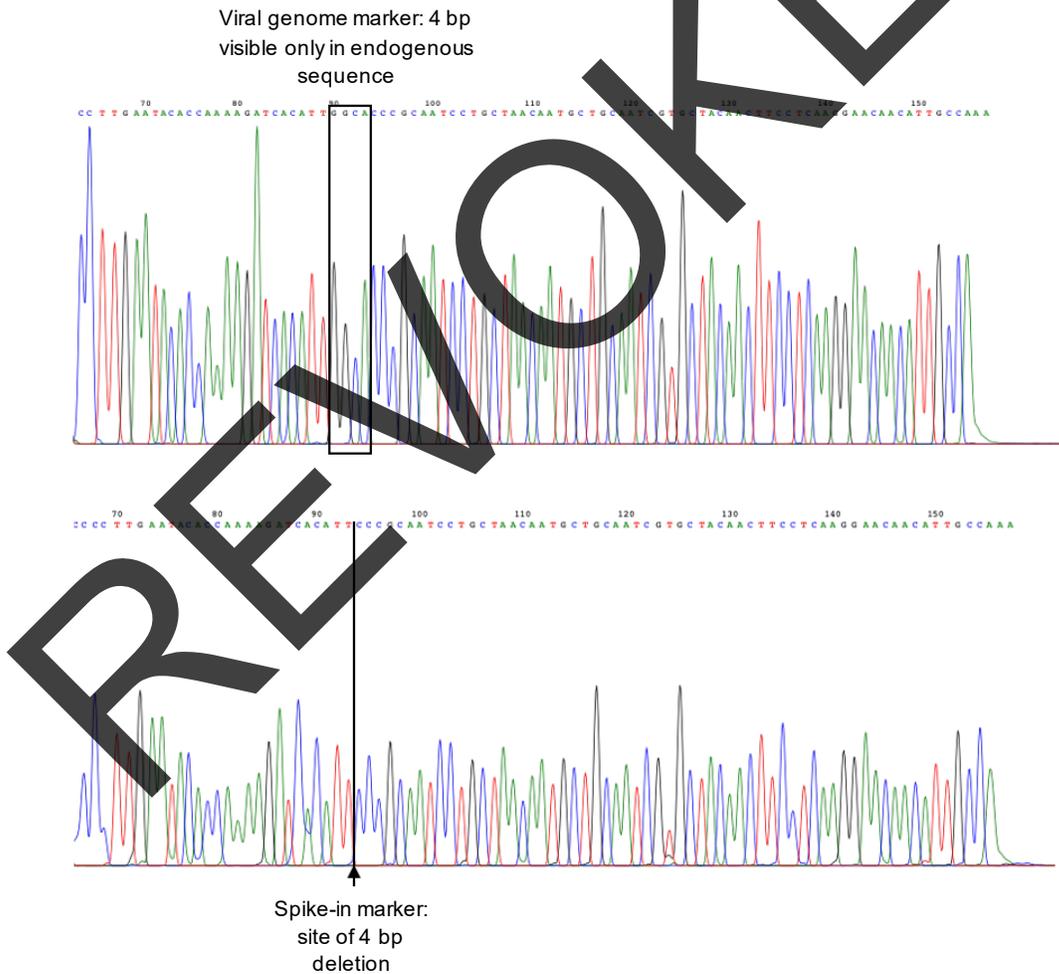


Figure 7 – Schematic distinguishing purely viral sequence (top) from purely spike-in sequence (bottom). Note the 4 missing bases in the bottom panel as compared to the top.

8.3. Result Interpretation

a. qSanger-COVID-19 Assay Controls – Positive, Negative and Internal

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted. Specifically, if the positive control is negative or invalid, the whole batch is reported as “INVALID”. If either of the two negative controls (No-RNA negative control or No-Template – No Spike-in control) is positive or invalid, the whole batch is reported as “INVALID”.

Expected performance of AccuPlex reference materials and negative and positive controls when valid

Control	Expected Values	
	SARS-CoV-2 (N)	Spike-In (IC)
No-RNA negative control	-	+
Positive Control: AccuPlex SARS-CoV-2 Positive Reference Material	+	+
No Template - No Spike-in Control	-	-

If any of the above controls do not exhibit the expected performance as described, the assay may have been improperly set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

If all controls have the expected results, the patient specimens will be reported out as “POSITIVE” when SARS-CoV-2 alignment is detected, “NEGATIVE” when only spike-in alignment is detected, or “INVALID” when neither SARS-CoV-2 nor spike-in alignment is detected (assay failure) or when signal-to-noise in chromatogram QC is not sufficiently high (sequencing failure) (Figure 2). “INVALID” specimens are retested once. If the retest result remains “INVALID”, then specimen recollection is recommended.

b. Examination and Interpretation of Patient Specimen Results:

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If the controls are not valid, the patient results cannot be interpreted.

SARS-CoV-2 (N)	Spike-In (IC)	Result Interpretation	Patient Report Verbiage
+	-/+*	SARS-CoV-2 RNA detected	SARS-CoV-2 RNA detected.
-	+	SARS-CoV-2 RNA NOT detected.	SARS-CoV-2 RNA NOT detected. Negative results do not preclude SARS-CoV-2 (COVID-19) infection and should not be used as the sole basis for treatment or other patient management decisions.
-	-	Results are invalid. Repeat testing If the result is still invalid, a new specimen should be obtained.	Invalid This specimen resulted in assay failure. The specimen might have contained an inadequate amount of clinical material. Repeat testing is required with a newly collected specimen.

*The absence of the internal spike-in control is acceptable in positive samples because under high viral load (i.e. >5000 molecules/reaction), the signal for viral RNA is so much greater than that for the spike-in internal control, that the control may not be readily observed. This is expected behavior and does not indicate any assay failure as long as there is observable viral sequence

The sequencing results must be manually inspected by trained personnel, to see if they align to both the spike-in and SARS-CoV-2 sequences. If the Sanger sequencing chromatogram aligns to the SARS-CoV-2 sequence alone, then this indicates that SARS-CoV-2 RNA was abundant in much higher level than the spike-in, and a POSITIVE result should be returned. If both SARS-CoV-2 and spike-in sequence alignments are found (mixed sequence), then SARS-CoV-2 RNA was present in the specimen at a comparable abundance to the spike-in, and as before, a POSITIVE result should be returned (weakly positive). If the spike-in alignment is recovered without a SARS-CoV-2 alignment (no final 4 bp tail), then SARS-CoV-2 RNA was not detected by the assay, and a NEGATIVE result is returned. If both spike-in and SARS-CoV-2 alignments are missing, then an assay failure occurred, and an INVALID result is returned.

PART 9. ASSAY LIMITATIONS

- Performance of the qSanger-COVID-19 Assay has only been established with nasopharyngeal swab specimens. Performance with other specimen types is unknown.
- Detection of SARS-CoV-2 RNA may be affected by sample collection methods, patient factors (e.g., presence of symptoms), and/or stage of infection.
- False-negative results may arise from:
 - Improper sample collection or handling

- Degradation of the SARS-CoV-2 RNA during shipping/storage
- Specimen collection after SARS-CoV-2 RNA can no longer be found in the specimen matrix
- Using unauthorized extraction or assay reagents
- The presence of RT-PCR inhibitors
- Mutation in the SARS-CoV-2 virus
- Failure to follow instructions for use
- Inadequate numbers of organisms are present in the sample
- False-positive results may arise from:
 - Cross contamination during specimen handling or preparation
 - Cross contamination between patient samples
 - Specimen mix-up
 - RNA contamination during product handling
- Negative results do not preclude infection with SARS-CoV-2 virus and should not be the sole basis of a patient management decision.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.
- Analyte targets (viral nucleic acid) may persist in vivo, independent of virus viability.
- Detection of analyte targets does not imply that the corresponding viruses are infectious or are the causative agents for clinical symptoms.
- Performance has not been established in asymptomatic individuals.

PART 10. CONDITIONS OF AUTHORIZATION FOR THE LABORATORY

The qSanger-COVID-19 Assay Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on FDA website: <https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas>.

However, to assist clinical laboratories using the qSanger-COVID-19 Assay, the relevant Conditions of Authorization are listed below.

- Authorized laboratories¹ using the qSanger-COVID-19 Assay will include with test result reports, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories using the qSanger-COVID-19 Assay will use the test as outlined in the authorized labeling. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use the qSanger-COVID-19 Assay are not permitted.

- Authorized laboratories that receive the qSanger-COVID-19 Assay will notify the relevant public health authorities of their intent to run the test prior to initiating testing.
- Authorized laboratories using the qSanger-COVID-19 Assay will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories will collect information on the performance of the qSanger-COVID-19 Assay and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and BillionToOne (Website: <https://billiontoone.com>, email: covid19support@billiontoone.com or by using <https://unityscreen.typeform.com/to/vNR12H>) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the qSanger-COVID-19 Assay of which they become aware.
- All laboratory personnel using the qSanger-COVID-19 Assay must be appropriately trained in RT-PCR and Sanger Sequencing techniques and use appropriate laboratory and personal protective equipment when handling this kit and use your test in accordance with the authorized labeling.
- BillionToOne Inc., its authorized distributor(s) and authorized laboratories using the qSanger-COVID-19 Assay will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

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

PART 11. ASSAY PERFORMANCE

11.1. Analytic Sensitivity and Limit of Detection (LOD)

The limit of detection was evaluated by spiking the Accuplex SARS-CoV-2 material (Seracare) into a pool of SARS-CoV-2 negative clinical NP swab matrix. The negative NP-swab pool was made from samples collected from individuals confirmed SARS-CoV-2 negative and were collected in viral transport media (VTM, Becton-Dickinson Viral Transport). A dilution series ranging from 50 copies/reaction (8000 copies/mL) to 4 copies/reaction (640 copies/mL) was prepared. Each concentration was tested with 20 replicates. The LOD was determined as the lowest concentration where the percentage of detected samples was 95% or above (Table 10). The LoD of the qSanger-COVID-19 Assay is 3200 copies/ml of sample.

Accuplex Copies/Reaction	Copies/ μ L of Sample	Detected/Tested	% Detected
50	8	20/20	100%
<u>20</u>	<u>3.2</u>	<u>19/20</u>	<u>95%</u>
10	1.6	14/20	70%
4	0.64	10/20	50%

11.2. Inclusivity

Spike-in sequences were designed using the viral genomic region approximately corresponding to the N3 region amplified by the CDC published N3 primer and probe sets. Spike-in molecules have sequences identical to SARS-CoV-2 sequence (LC528232) including base positions 28216 to 29280 but lacking 4 bases 28715-28718, in order to create a frameshift that can be detected in data analysis. Primers that co-amplify both SARS-CoV-2 and spike-in were used for amplification. Sequencing was performed using a nested forward primer to increase specificity in human specimens.

An *in silico* analysis of the test's primer binding sequences was performed with 4635 SARS-CoV-2 full-length sequences deposited in NCBI. Of these sequences, more than 99% of sequences are identical to the reverse primer and 98.5% are identical to the forward primer. 1.4% of sequences exhibit single SNPs in at position 5 from the 5' end of the forward primer, accounting for a homology of 95.5%. Given the location of this SNP and the limited impact on melting temperature of the primers, it is anticipated that this SARS-CoV-2 sequence would still be detected by this assay. 98.5% of the sequences have predicted melting temperatures greater than or equal to the annealing temperature of the thermocycling reaction.

11.3. Cross-reactivity

An *in silico* analysis of the test primer sequences was performed with the following organisms:

Table 1 In silico analysis

Accession	Description	F Primer % Homology (n/total bases)	R Primer % Homology (n/total bases)
NC_002645.1	Human coronavirus 229E, complete genome	55% (12/22)	52% (12/23)
NC_006213.1	Human coronavirus OC43 strain ATCC VR-759, complete genome	41% (9/22)	43% (10/23)
NC_006577.2	Human coronavirus HKU1, complete genome	45% (10/22)	48% (11/23)
NC_005831.2	Human Coronavirus NL63, complete genome	45% (10/22)	52% (12/23)
NC_004718.3	SARS coronavirus Tor2, complete genome	91% (20/22)	100% (23/23)

NC_019843.3	Middle East respiratory syndrome coronavirus, complete genome	64% (14/22)	43% (10/23)
AC_000017.1	Human adenovirus type 1, complete genome	41% (9/22)	39% (9/23)
NC_039199.1	Human metapneumovirus isolate 00-1, complete genome	50% (11/22)	39% (9/23)
NC_003461.1	Human parainfluenza virus 1, complete genome	41% (9/22)	39% (9/23)
NC_003443.1	Human rubulavirus 2, complete genome	41% (9/22)	39% (9/23)
NC_001796.2	Human parainfluenza virus 3, complete genome	41% (9/22)	52% (12/23)
NC_021928.1	Human parainfluenza virus 4a viral cRNA, complete genome, strain: M-25	36% (8/22)	43% (10/23)
NC_026423.1	Influenza A virus (A/Shanghai/02/2013(H7N9)) segment 2 polymerase PB1 (PB1) and PB1-F2 protein (PB1-F2) genes, complete cds	50% (11/22)	52% (12/23)
NC_002204.1	Influenza B virus RNA 1, complete sequence	36% (8/22)	39% (9/23)
NC_006309.2, NC_006308.2	Influenza C virus (C/Ann Arbor/1/50) (all accessions)	45% (10/22)	43% (10/23)
NC_038308.1	Human enterovirus 68 strain Fermon, complete genome	41% (9/22)	39% (9/23)
NC_001803.1	Respiratory syncytial virus, complete genome	50% (11/22)	39% (9/23)
NC_009996.1	Human rhinovirus C, complete genome	41% (9/22)	43% (10/23)
NC_005043.1	<i>Chlamydia pneumoniae</i> TW-183, complete sequence	50% (11/22)	57% (13/23)
NZ_QQLA01000 002.1/NZ_MZJN0 1000009.1	<i>Haemophilus influenzae</i> strain M14791 M14791_HUY4654A129_cleaned_ctg_921, whole genome shotgun sequence/ <i>Haemophilus influenzae</i> strain 48P45H1 N48P45H1_11_8, whole genome shotgun sequence	59% (13/22)	57% (13/23)
NZ_QFHP010000 13.1/NZ_QFHP01 000039.1	<i>Legionella pneumophila</i> strain HH56 NODE_13_length_107514_cov_50.6576, whole genome shotgun sequence/ <i>Legionella pneumophila</i> strain HH56 NODE_39_length_871_cov_120.831, whole genome shotgun sequence	64% (14/22)	61% (14/23)
No Similarity Found (taxid:1773)	<i>Mycobacterium tuberculosis</i> (taxid:1773)	NA	NA
NZ_CGVP010000 16.1	<i>Streptococcus pneumoniae</i> strain SMRU22, whole genome shotgun sequence	64% (14/22)	65% (15/23)
NZ_CAAINE010 000002.1/NZ_CA AHYZ010000009. 1	<i>Streptococcus pyogenes</i> strain NS678, whole genome shotgun sequence/ <i>Streptococcus pyogenes</i> strain 31089V2S1, whole genome shotgun sequence	64% (14/22)	61% (14/23)
NZ_CSNY010001 65.1	<i>Bordetella pertussis</i> strain B082 isolate 1977/3, whole genome shotgun sequence	55% (12/22)	57% (13/23)
NZ_BLHG010000 07.1	<i>Mycoplasma pneumoniae</i> strain KPI-131 contig_7, whole genome shotgun sequence	50% (11/22)	52% (12/23)
NW_017264788.1	<i>Pneumocystis jirovecii</i> RU7 chromosome Unknown supercont1.14, whole genome shotgun sequence	59% (13/22)	91% (20/22)
NC_032090.1/NC 032096.1	<i>Candida albicans</i> SC5314 (all accessions)	68% (15/22)	61% (14/23)

NZ_CAADQY010000466.1	<i>Pseudomonas aeruginosa</i> isolate XDR-PA, whole genome shotgun sequence	68% (15/22)	65% (15/23)
NZ_CP035288.1	<i>Staphylococcus epidermidis</i> strain ATCC 14990 chromosome, complete genome	59% (13/22)	57% (13/23)
NZ_PKHZ01000004.1/NZ_WMYP01000001.1	<i>Streptococcus salivarius</i> strain UMB0028 .21837_8_51.4, whole genome shotgun sequence/ <i>Streptococcus salivarius</i> strain BIOML-A3 scaffold1 size599083, whole genome shotgun sequence	59% (13/22)	70% (16/23)
NC_001897.1	Human parechovirus, genome	45% (10/22)	43% (10/23)
NZ_BEYJ01000010.1/NZ_PSZX01000019.1	<i>Staphylococcus aureus</i> strain GUATP 151, whole genome shotgun sequence/ <i>Staphylococcus aureus</i> strain SKY9-1 SKY9-1_R1_(paired)_contig_19, whole genome shotgun sequence	64% (14/22)	65% (15/23)
NC_007530.2	<i>Bacillus anthracis</i> str. 'Ames Ancestor', complete sequence	55% (12/22)	61% (14/23)
NC_014147.1	<i>Moraxella catarrhalis</i> BBH18, complete genome	55% (12/22)	57% (13/23)
NZ_CP031252.1	<i>Neisseria elongata</i> strain M15911 chromosome, complete genome	64% (14/22)	52% (12/23)
NZ_OAAT01000028.1/ NZ_OAAT01000003.1	<i>Neisseria meningitidis</i> strain <i>Neisseria meningitidis</i> isolate R575, whole genome shotgun sequence	64% (14/22)	65% (15/23)
NZ_RQHK01000017.1/NZ_NPEI01000001.1	<i>Leptospira</i> sp. (all accessions: taxid:171)	64% (14/22)	74% (17/23)
NC_017287.1	<i>Chlamydia psittaci</i> 6BC, complete sequence	55% (12/22)	70% (16/23)
NC_002971.4	<i>Coxiella burnetii</i> RSA 493, complete genome	64% (14/22)	70% (16/23)

Among the tested organisms, only SARS-coronavirus (SARS-CoV) exhibited more than 80% homology for the primer sequences. The forward primer had 91% homology (corresponding to 2 mismatches) and the reverse primer exhibited 100% homology.

However, because this assay sequences the internal sequence of the amplicon, the assay is able to distinguish SARS-CoV from SARS-CoV-2 based on the 4 SNPs in the internal control sequence that differentiate these two sequences. Moreover, as SARS-coronavirus is not currently circulating in the population, any cross-reactivity is not expected to result in false positives.

BLAST analysis indicated all other species had less than 80% homology in both amplification primers with the exception of *Pneumocystis jirovecii* which exhibited 91% homology in the reverse primer but only 59% homology in the forward primer. These primers are separated by 20,000 base-pairs and as such extremely unlikely to result in an amplification product that could also be sequenced by the qSanger-COVID-19 Assay. In addition, the intervening sequence is not homologous to SARS-CoV-2 and because the test is sequencing based and thereby identifies the specific organism, no false positive result would be generated.

11.4. Interfering Substances

The qSanger-COVID-19 Assay does not require RNA purification and therefore, potentially interfering substances commonly found in NP swab samples were tested for potential interference. The indicated final concentration of each substance was added to pooled negative clinical sample matrix and samples were tested in the absence and presence of 2x LoD of Accuplex SARS-CoV-2 material (Seracare). All conditions were tested in triplicate and the results analyzed for detection of COVID19. Results are summarized below.

Table 2 Interfering substances study

Substance	Concentration	Detected/ Tested	
		Negative	Positive
Afrin	10% v/v	0/3	3/3
Blood	5% v/v	0/3	3/3
Cepacol	5 mg/mL	0/3	3/3
Flonase	5% v/v	0/3	3/3
Mucin	2.5 mg/mL	0/3	3/3
Mupirocin	5 mg/mL	0/3	3/3
Tamiflu	2.2 µg/mL	0/3	3/3
Tobramycin	4 µg/mL	0/3	3/3
Matrix control	NA	0/3	3/3

11.5. Clinical Evaluation:

For the clinical validation, 30 SARS-CoV-2 positive Nasopharyngeal Swabs and 30 SARS-CoV-2 negative Nasopharyngeal Swabs collected in Becton Dickinson Universal Viral Transport (specifically these Catalog #'s BD 220527, 220529 and 220531) were tested. The samples were collected during standard clinical visits at an academic medical center and had prior SARS-CoV-2 RT-PCR results obtained with EUA authorized RT-PCR tests.

The qSanger-COVID-19 Assay for clinical validation was performed by an independent laboratory. Raw data was analyzed, and outcome determined at BillionToOne. Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA) were calculated in comparison to the prior result with the FDA authorized test.

<i>NP swabs</i>		EUA authorized Comparator Assays		Total
		POSITIVE	NEGATIVE	
qSanger- COVID-19 Assay	POSITIVE	27	0	27
	NEGATIVE	3*	30**	33
Total		30	30	60

* The missed samples were retested on an additional EUA-authorized test and had the following Ct values with that test: for the S-Gene: 31.8, 31.3, and 33.0; the N-gene: 29.3, 30.8, and 33.0; and ORF1Ab: 29.7, 29.3, and 29.6. The mean Cts of this additional EUA authorized test at its LoD for NP swab are 34.3 (S), 29.1 (N) and 30.7 (ORF1ab), indicating that all three samples were low positive samples and therefore likely below the LoD of the qSanger-COVID-19 Assay.

**4 out of 30 samples had assay failure (no sequence present in .ab1 file, i.e., invalid results), and upon repeat, they all resulted in negative calls.

PPA: $27/30 = 90\%$ (95% CI: 74.4 – 96.5%)

NPA: $30/30 = 100\%$ (95% CI: 88.7 – 100%)

11.6. FDA SARS-CoV-2 Reference Panel Testing:

The evaluation of sensitivity and MERS-CoV cross-reactivity was performed using reference material (T1), blinded samples and a standard protocol provided by the FDA. The study included a range finding study and a confirmatory study for LoD. Blinded sample testing was used to establish specificity and to corroborate the LoD. The samples were tested with the q-Sanger COVID-19 Assay in accordance with the Instructions for Use. The results are summarized in Table 14.

Table 14: Summary of LoD Confirmation Result Using the FDA SARS-CoV-2 Reference Panel

Reference Materials Provided by FDA	Specimen Type	Product LoD	Cross-Reactivity
SARS-CoV-2	Nasopharyngeal Swab	5.4×10^4 NDU/mL	N/A
MERS-CoV		N/A	ND

NDU/mL: RNA NAAT detectable units/mL

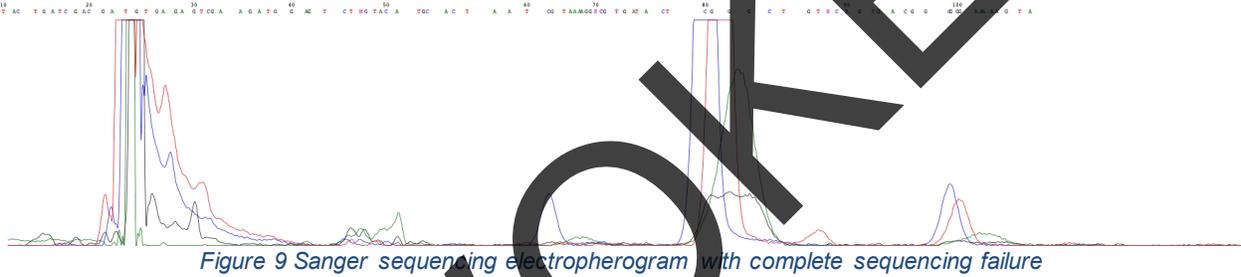
N/A: Not Applicable

ND: Not Detected

Dye Blobs	
Possible Cause	Recommended Action(s)
Insufficient dye-terminator clean-up	If possible, repeat dye-terminator clean-up, being sure to execute proper wash steps.
	If the cycle sequenced product is no longer stable and/or available, repeat cycle sequencing and dye-terminator clean-up.

Complete Sequencing Failure

The following trace is an example of complete sequencing failure. In this example, the only peaks that are present are dye blobs, suggesting that this failure was likely due to issues with the cycle sequenced product that was loaded.



Complete Sequencing Failure	
Possible Cause	Recommended Action(s)
Wrong sequencing primer was used for cycle sequencing.	Repeat cycle sequencing. Ensure proper sequencing primer is used.
Insufficient amount of template was loaded.	Resequence with more template.
	Quantify DNA produced from the RT-PCR reaction to check amplification.
	Quantify DNA post RT-PCR clean-up, as product could have been lost during purification.
	If necessary, repeat the RT-PCR amplification failing sample(s).

<p>Suboptimal cycle sequencing reaction conditions, causing cycle sequencing failure.</p>	<p>Repeat cycle sequencing with modified conditions, ensuring:</p> <ol style="list-style-type: none"> 1. Sufficient template is loaded 2. Dye-terminator is of sufficient concentration 3. Annealing temperature is appropriate for sequencing primer (50° C) 4. Cycle count is appropriate for cycle sequencing 5. Denaturation, annealing, and extension times are all of appropriate length
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Loss of Resolution

Loss of resolution in capillary electrophoresis is characterized by broader peaks and the loss of ability to distinguish two consecutive bases of the same type. Loss of resolution of the peaks can affect the ability of the COVID-19 qSanger algorithm to result samples. Resolution loss that causes peaks to be indistinguishable, such as that seen in Figure 5, result in analysis failure.

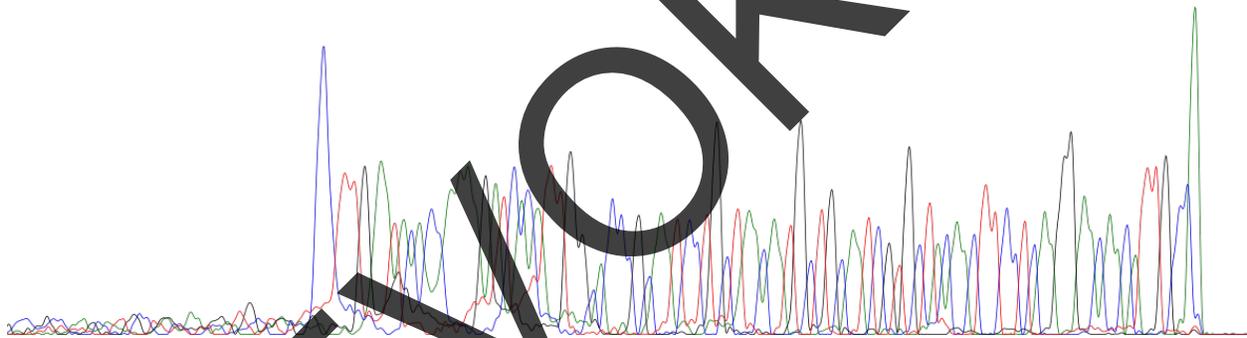


Figure 10 Electropherogram with bad resolution loss, causing analysis failure

Data can still be usable despite resolution loss if peaks are distinguishable. An example of such tolerable resolution loss can be seen below in Figure 11.

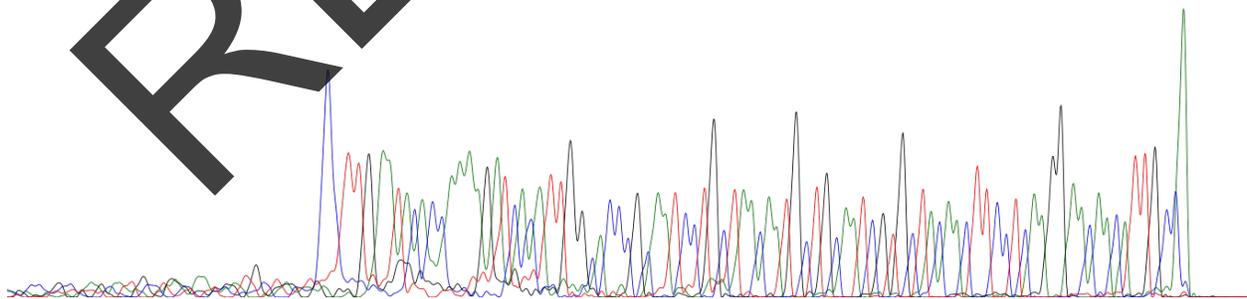


Figure 11 Electropherogram with tolerable resolution loss

Loss of Resolution	
Possible Cause	Recommended Action(s)
Polymer degradation	Check that the polymer was properly stored is not expired. If necessary, obtain fresh polymer.
	Perform appropriate maintenance according to the instrument user manual.
	Resequence samples once the machine has been maintained.
Buffer expiration	Check that the 1X Running Buffer was properly stored and is not expired. If necessary, prepare fresh 1X Running Buffer.
	Replace running buffer according to the instrument user manual.
	Resequence samples once the machine has been maintained.
Capillary array degradation or damage	Perform appropriate maintenance according to the instrument user manual.

Random Extended Sequence

Since this assay is run without PCR stop, random extended sequence is sometimes visible. This extended sequence can interfere with the qSanger COVID-19 algorithm. An example of a trace with this random extended sequence is included below in Figure 12.

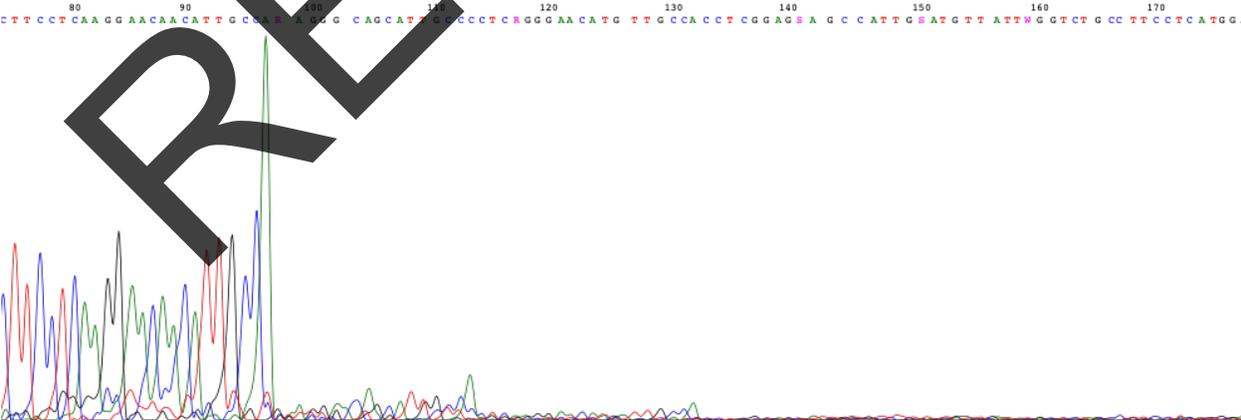


Figure 12 Electropherogram with random extended sequence

Random Extended Sequence	
Possible Cause	Recommended Action(s)
Insufficient amount of template was loaded.	Resequence with more template.
	Quantify DNA produced from the RT-PCR reaction to check amplification.
	Quantify DNA post RT-PCR clean-up, as product could have been lost during purification.
	If necessary, repeat the qSanger COVID-19 RT-PCR for the failing sample(s).

Truncated Sequences

Electropherograms with truncated sequence, such as that shown below in Figure 13, contain random, noisy sequence often indicate reaction failure.

T C G G T T Y W S A A M Y A G C C T A G T C A C Y K C T G A A A T T G A T G A T G A T G C C T C T T A

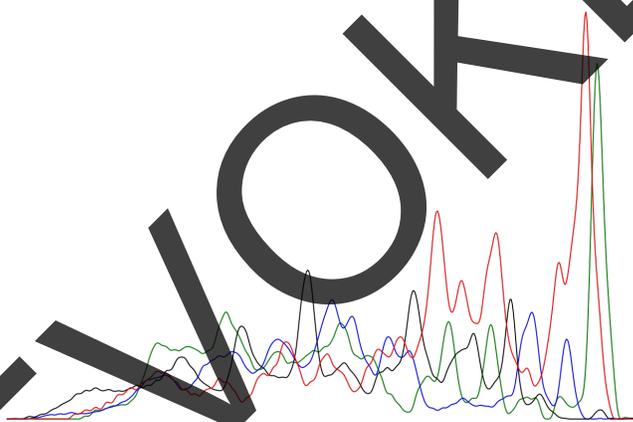


Figure 13 Electropherogram with truncated sequence

Truncated Sequence, General	
Possible Cause	Recommended Action(s)
Wrong sequencing primer was used for cycle sequencing.	Repeat cycle sequencing. Ensure proper sequencing primer is used.
Poor amplification or off-target amplification product.	Repeat the RT-PCR amplification for failing sample(s).

There is also a specific type of truncated sequence, depicted in Figure 14, which is commonly seen in failed RT-PCR reactions. The example shown in Figure 14 differs from that shown above (Figure 13) because the sequence at the 3' end is consistent with the sequence of the reverse primer used for RT-PCR. In the example, the signal from the amplicon with the reverse primer

was sufficient to be called by the basecaller. Note that the signal from the reverse primer may not be strong enough to be called by the basecaller but should be visible by manual inspection.

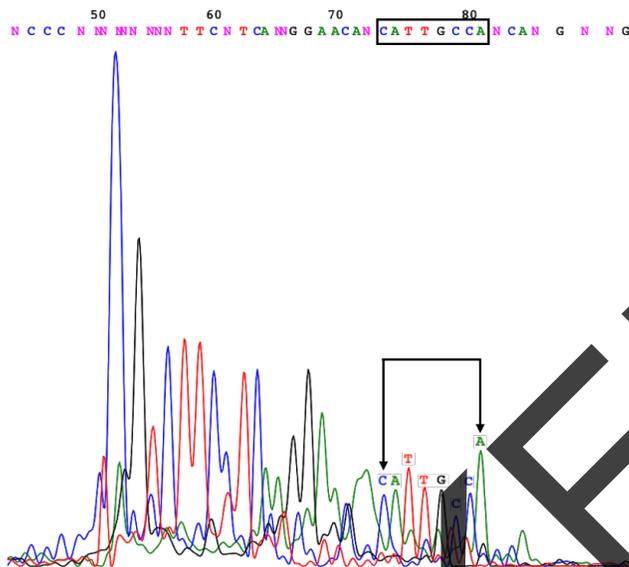


Figure 14 Truncated sequence with visible RT-PCR reverse primer sequence

Truncated Sequence, Visible Signal from Reverse RT-PCR Primer	
Possible Cause	Recommended Action(s)
Amplification failure: amplification of off-target product	See recommended actions for “Gel electrophoresis shows dominant band at around 50 bp...” on page 12.

Additional Quality Control and Troubleshooting Steps

As described in the previous section, Sanger sequencing will identify many process failures. Thus, additional performing quality control (QC) steps prior to sequencing is not typically required. However, to better diagnose process failures, performing the following troubleshooting steps post RT-PCR is recommended.

DNA Quantification

Perform DNA quantification on the samples to determine the concentration of DNA product in the reactions. A normal range is 10-25 ng/μL.

Methods: Qubit Fluorometer (Thermo Fisher), NanoDrop (Thermo Fisher), or similar

Gel Electrophoresis

Perform gel electrophoresis on the samples to determine the amplicon size(s) of the DNA products in the reactions. See Figure 15 for typical gel electrophoresis results and interpretations.

Methods: traditional agarose gel electrophoresis, TapeStation (Agilent), or similar

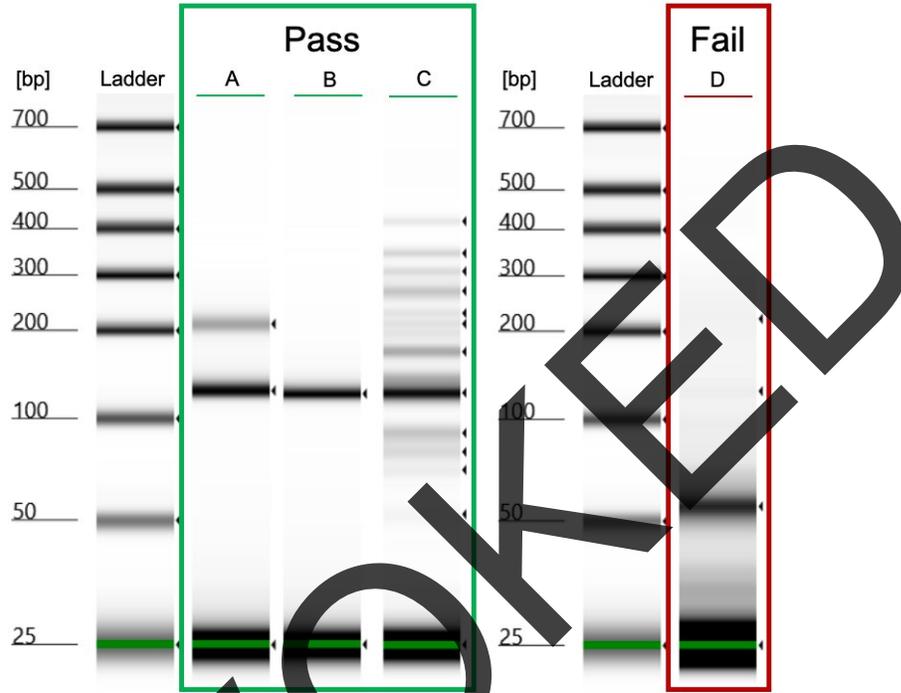


Figure 15 Typical gel electrophoresis images (taken by TapeStation) for RT-PCR products. Double banding, with a dominant band around 125 bp, (A) is commonly seen in positive control samples. A single band around 125 bp (B) is expected for negative control samples

Possible causes of RT-PCR Failure

RT-PCR Failure		
Observation	Possible Cause	Recommended Action(s)
<i>Amplification Failure</i>		
DNA quantification and/or gel electrophoresis shows no DNA is present in the sample. <i>Note: all samples, including water-only samples, should result in DNA product due to the spike-in.</i>	Enzyme A3 degraded.	Ensure Enzyme A3 was stored properly (at -20°C).
		Do not vortex Enzyme A3.
		Either: Assess activity of enzyme by

		<p>repeating the qSanger COVID-19 test, using fresh nuclease-free water as sample input.</p> <p>Or: Discard previously-used Enzyme A3 and obtain fresh stock. Then, repeat the qSanger COVID-19 test for all samples.</p>
	Enzyme A3 was missing from the <i>Assembled Master Mix</i> .	<p>Enzyme A3 appears blue in color. Reactions should similarly appear blue in color.</p> <p>Repeat the qSanger COVID-19 test for all samples. Be sure to add all components to the <i>Assembled Master Mix</i>.</p>
	Reagent A1 was missing from the <i>Assembled Master Mix</i> .	Repeat the qSanger COVID-19 test for all samples. Be sure to add all components to the <i>Assembled Master Mix</i> .
	Thermal cycler failure.	<p>Ensure that the instrument is calibrated and operating within specifications.</p> <p>Ensure that the correct thermal cycler program is selected.</p>
Gel electrophoresis shows dominant band at around 50 bp. Target band (around 125 bp) is either not dominant or not present.	Inefficient heat transfer	<p>Test different consumables.</p> <p><i>Note: In house, we were able to rescue samples by re-running reactions in strip tubes at the same reaction volume.</i></p>
		<p>Reduce reaction volume to 25 μL. Maintain proportional sample and reagent input.</p> <p><i>Note: In house, reducing the reaction volume to 25 μL was sufficient to rescue failed sample reactions conducted in PCR plates.</i></p>
	Thermal cycler failure.	Ensure that the instrument is calibrated and operating within specifications.

		Ensure that the correct thermal cycler program is selected.
<i>Reverse Transcriptase (RT) Failure</i>		
Positive control samples test negative but DNA quantification confirms and/or gel electrophoresis DNA is present in all samples.	RNase contamination	Be sure to thoroughly clean workspace and gloves with RNase decontaminant (RNase away, RNaseZap, or similar)
		Be sure to use only RNase free consumables (conical and microcentrifuge tubes, pipette tips, PCR plates and plate seals).
		Discard any reagents that may have been contaminated with RNase.
	Enzyme A2 Degraded	Ensure Enzyme A2 was stored properly (at -20°C).
		Do not vortex Enzyme A2.
		Either: Assess activity of enzyme by repeating the qSanger COVID-19 test, using a fresh set of controls as sample input.
		Or: Discard previously-used Enzyme A3 and obtain fresh stock. Then, repeat the qSanger COVID-19 test for all samples.
Enzyme A2 was missing from the <i>Assembled Master Mix</i> .	Repeat the qSanger COVID-19 test for all samples. Be sure to add all components to the <i>Assembled Master Mix</i> .	
Sample was not added to the reaction plate.	Repeat the qSanger COVID-19 test for all samples. Be sure to add all samples to their respective wells.	

PART 13. CUSTOMER AND TECHNICAL SUPPORT

For customer service and technical support, please contact

BillionToOne, Inc.
1035 O'Brien
Menlo Park, CA USA 94025
+1-844-987-1361.
Email: covid19support@billiontoone.com

PART 14. SYMBOLS USED IN PACKAGING

	“Batch code” or “Lot number”
	“Temperature limitation.” The upper and lower temperature limits will be indicated on either side of the symbol.
	“Use By.” This symbol is intended to indicate that the kit should not be used after the end of the year and month shown.
	“Reference Number” or Manufacturer’s catalog number. This number can be used for reordering.

PART 15. REFERENCES

- BigDye™ Terminator v3.1 Cycle Sequencing Kit USER GUIDE ([link](#))
- 3730xl DNA Analyzer USER GUIDE ([link](#)) [for “latest version”, catalog number A41046]

For use under Emergency Use Authorization Only

**For Prescription Use Only.
For *in vitro* diagnostic (IVD) use**

Consult instructions for use. Electronic Instruction for Use (IFU) is available via a linked URL on our website BillionToOne.com or directly here: <https://www.dropbox.com/s/tg5wa5mlfrcv2h2/EUA201022.IFU.BillionToOne.pdf?dl=1>

Intended use

The qSanger-COVID-19 Assay is a Sanger sequencing-based RNA extraction-free diagnostic test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory swab specimens (such as nasal swab, mid-turbinate swab, nasopharyngeal swab, and oropharyngeal swab specimens) from individuals suspected of COVID-19 by their healthcare provider. Testing is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, and meet requirements to perform high complexity tests.

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

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

The qSanger-COVID-19 Assay is intended for use by qualified clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR, Sanger Sequencing and *in vitro* diagnostic procedures. The qSanger-COVID-19 Assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

Principle

The qSanger-COVID-19 Assay is a Sanger sequencing-based test for detection of SARS-CoV-2 RNA. The qSanger-COVID-19 Assay is designed to detect RNA from SARS-CoV-2 in nasopharyngeal and oropharyngeal swabs from patients who are suspected of COVID-19. Patient samples are tested directly without extraction. VTM from Nasopharyngeal (NP)/oropharyngeal (OP) swabs is added directly to a reverse transcription and an endpoint PCR reaction. The amplification product of this reaction is then sequenced using a modified Sanger sequencing approach. Detection of the correct sequencing involves use of a frame-shifted spike-in sequence that serves as an internal control for each sample and is described in detail in the Instructions For Use document.

Materials Provided and Storage

Table 1. BillionToOne, Inc. qSanger-COVID-19 Assay

Component (for 2000 reactions, with 20% overage)	Volume	Shipping	Storage
Reagent A1: Sars-CoV-2 Primer and Spike-In Reagent	12 mL	Dry ice	-20°C (-15 to -25°C)
Enzyme A2: Luna® WarmStart® RT Enzyme Mix (20x)	3 mL		
Enzyme A3: Luna® Universal Probe One Step Reaction Mix No ROX (2x)	30 mL		
Reagent B1: Sanger Sequence Primer	5 mL		
Reagent C1: SARS-CoV-2 Primer Mix (No Spike-In)	0.2 mL		

Warnings and Precautions

- For *in vitro* diagnostic use
- For prescription use only
- For use under FDA emergency use authorization (EUA) only
- The qSanger-COVID-19 Assay has not been FDA cleared or approved;
- The qSanger-COVID-19 Assay has been authorized by FDA under an Emergency Use Authorization (EUA) for use by laboratories, which are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, and meet the requirements to perform high complexity tests;
- The qSanger-COVID-19 Assay has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.
- The qSanger-COVID-19 Assay is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Only personnel proficient in handling infectious materials and trained in the use of the qSanger-COVID-19 Assay should perform this procedure.
- RNA is highly unstable and extra precaution should be employed to ensure it does not degrade in samples.
- Maintain separate areas for sample accessioning, RT-PCR and the set-up of the sequencing reaction to minimize the risk of contamination with amplifiable material in the sample processing steps.
- The performance of the qSanger-COVID-19 Assay was established using nasal swab specimens. Performance with other specimen types has not been established.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

- Laboratory personnel must be familiar with the protocol and all instrumentation used.
- Maintain dedicated equipment (e.g., pipettes, microcentrifuges, vortexes) and supplies (e.g., 1.5mL tubes, pipette tips, gloves) for assay reagent setup and handling of extracted nucleic acids.
- Workflow must always be in the direction of clean area to dirty area.
- Carefully clean pipettes, containers, and surfaces with RNaseAway, RNaseZap, or a similar product before and between sample batches. Change gloves frequently.
- Never bring extracted nucleic acid or amplification products into the assay set-up area.
- Use nuclease-free, aerosol barrier (filter) pipette tips only.

Symbols

Table 2. Symbols and Description

Symbol	Description
	"Batch code" or "Lot number"
	"Temperature limitation." The upper and lower temperature limits will be indicated on either side of the symbol.
	"Use By" This symbol is intended to indicate that the kit should not be used after the end of the year and month shown.
	"Reference Number" or Manufacturer's catalog number. This number can be used for reordering.

References

- BigDye™ Terminator v3.1 Cycle Sequencing Kit USER GUIDE ([link](#))
- 3730xl DNA Analyzer USER GUIDE ([link](#)) [for "latest version", catalog number A41046]

Contact

Manufacturer

BillionToOne, Inc.
 1035 O'Brien Drive
 Menlo Park, CA 94025 USA
 Tel: +1 844 987 1361
 Fax: +1 833 915 0146
 Technical/Customer Support email: covid19support@billiontoone.com

Visit our website: www.billiontoone.com