MiRXES Fortitude SARS-CoV-2 & FluA/B Test

Instructions for Use

Instructions for Use

IVD

REF FGS0022-2

Σ 200

Important!

The instructions for use must be read carefully prior to use and followed strictly to achieve reliable results. Any deviations from the instructions will have a significant impact on the end result.

Storage and Transportation Conditions

-15°C

-25°C

Protect from light during transportation and storage.

MiRXES Pte Ltd
2 Tukang Innovation Grove
JTC MedTech Hub
#09-02
Singapore 618305

For prescription use only.
For in vitro diagnostic use only.

TABLE OF CONTENTS

1 Introduction

Purpose

This document describes the use of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test, a real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay, for the qualitative detection of SARS-CoV-2, Influenza A, and/or Influenza B specific RNA in nasopharyngeal/oropharyngeal swabs, nasopharyngeal wash/aspirate, nasal wash/aspirate and anterior/mid-turbinate nasal swabs.

Intended Use

MiRXES Fortitude SARS-CoV-2 & FluA/B Test is a real-time RT-PCR intended for the qualitative detection of RNA from SARS-CoV-2, Influenza A, and/or Influenza B in nasopharyngeal/oropharyngeal swabs, nasopharyngeal wash/aspirate, nasal wash/aspirate and anterior/mid-turbinate nasal swabs samples from patients suspected of COVID-19 by a healthcare provider. Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories. This test is for in vitro diagnostic use only and for prescription use only. This test is not intended to be used for PoC or in Near Patient settings.

The RNA from SARS-CoV-2, Influenza A, and Influenza B is generally detectable in upper respiratory during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2, Influenza, and/or Influenza B 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 test results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2, Influenza A and/or Influenza B 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 MiRXES Fortitude SARS-CoV-2 & FluA/B Test is intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures. This test has not been reviewed by the Food and Drug Administration.

Read the Instructions for Use carefully before using this product.

2 Principle of the Procedure

Nucleic acids are isolated and purified from the specimens using QIAamp Viral RNA Mini Kit following extraction producedures provided in manufacturer's instruction. The purified nucleic acid is reversed-transcribed using Probes Reaction Mix/Reverse Transcriptase into cDNA which is then subsequently amplified in Bio-Rad CFX96 Real-Time PCR System or Applied Biosystems 7500 Fast Real-Time PCR System. During the process, the probes anneal to specific target sequences located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe and release the quencher. The separation of quencher from the reporter dye will then generate a fluorescent signal. With each cyle, additional reporter dye molecules are cleaved from their respective probes and increase the fluorescence intensity. The fluorescence intensity is monitored at each PCR cycle by Bio-Rad CFX96 Real-Time PCR System or Applied Biosystems 7500 Fast Real-Time PCR System. The instrument will differentiate between the signal by detecting viral-specific probes at different wavelength.

3 Product Description

- MiRXES Fortitude SARS-CoV-2 & FluA/B Test contains assays and controls for the qualitative detection of SARS-CoV-2 specific RNA, Influenza A specific RNA and Influenza B specific RNA, via an one-step, multiplex real-time RT-PCR reaction run in a single well for each sample. The test also contains primers and probe to detect the human RNase P (RP) in clinical specimens. The oligonucleotide primers and probes for detection of SARS-CoV-2 were selected from an evolutionarily conserved region of the ORF1ab gene and N gene. Primers and probes for the detection of influenza A viruses were selected from an evolutionarily well conserved region of the Matrix Protein gene. The primers and probe selected for detection of influenza B viruses were selected from a conserved region of the Matrix Protein gene.

- The MiRXES Fortitude SARS-CoV-2 & FluA/B Test was co-developed with the Singapore Agency of Science Technology and Research (A*STAR)'s Experimental Drug Development Centre (EDDC), Bioinformatics Institute (BII), Diagnostics Development (DxD) Hub and the Department of Laboratory Medicine at Tan Tock Seng Hospital (TTSH).

- The MiRXES Fortitude SARS-CoV-2 & FluA/B Test contains five unique sets of forward primers, reverse primers and probes, pre-mixed in Fortitude CoVFluA/B Test Primers-Probes Mix (Kit Component 6)
    - ORF1ab region (probe with FAM fluorophore) of SARS-CoV-2 genome
    - N region (probe with FAM fluorophore) of SARS-CoV-2 genome
    - Matrix protein region (probe with Hex fluorophore) of Influenza A genome
    - Matrix protein region (probe with Texas Red fluorophore) of Influenza B genome
    - Human RNase P region (probe with Cy5 fluorophore) of Human genome

- The MiRXES Fortitude SARS-CoV-2 & FluA/B Test contains two different controls
    - **Fortitude CoVFluA/B Test Negative Control** is used to confirm the absence of contamination throughout the test process from viral RNA isolation to RT-PCR amplification.
    - **Fortitude CoVFluA/B Test Positive Control** consists of synthetic RNA of the SARS-CoV-2 genome, Influenza A genome, Influenza B genome and Human RNase P (Rp) gene segment. It is provided at high concentration and required a dilution before adding into the test. The positive control does not participate in the extraction process but should be directly added into the PCR reaction solution.
- The endogenous Human RNase P is used as an internal control and extraction control to monitor sampling quality and the entire sample processing procedure.
- Each MiRXES Fortitude SARS-CoV-2 & FluA/B Test contains the following 6 components in 10 vials. Each kit is sufficient for 200 reactions.

Table 1: Kit Components

Component Label	Part Number	Component	Number of Vials	Volume [µL/Vial]
1	INT0076	Nuclease Free Water	2	1200
2	INT0071	Positive Control	2	20
3	INT0072	Negative Control	1	1200
4	INT0073	Probes Reaction Mix	3	920
5	INT0074	Reverse Transcriptase	1	110
6	INT0075	Primers - Probes Mix	1	220

NOTE: ***To avoid degradation of positive controls, users are advised to make small aliquots and avoid multiple freeze-thaw.***

4 Storage and Transportation

- The MiRXES Fortitude SARS-CoV-2 & FluA/B Test is shipped in a cold chain environment. The components of the kit should arrive cold. If the kit components are not cold upon receipt, or if vials have been compromised during shipment, contact Technical Support (refer to section 2) for assistance.
- All components are to be stored between -25°C and -15°C upon arrival.
- Always check the expiration date prior to use. Do not use reagents beyond the specified expiration date.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), positive control (stock and/or diluted) and enzyme master mix must be thawed and kept on ice at all times during preparation and use.

5 Material and Devices Required But Not Provided

NOTE: *The names of vendors or manufacturers are provided as examples of suitable product sources. Users have to conduct verification whether using product sources provided or other product sources.*

- 1.5 mL polypropylene microcentrifuge tubes (DNase/RNase free), 2 mL polypropylene microcentrifuge tubes (DNase/RNase free) and/or 5 mL polypropylene microcentrifuge tubes (DNase/RNase free).
- Racks for 1.5 mL/2 mL/5 mL microcentrifuge tubes.
- Molecular-grade nuclease-free water.
- 96-well cold blocks .
- Multichannel or single channel Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL).
- Disposable pipette tips with filters and aerosol barriers.
- Disposable powder-free gloves.
- Micropipettors (1-10 µL, 10-200 µL).
- QIAamp Viral RNA Mini Kit (Cat No.: 52904 or 52906)
- Appropriate real-time PCR instrument:
    - Applied Biosystems™ 7500 Fast Real-Time PCR System (Cat No.: 4351106); or
    - Bio-Rad CFX96 Real-Time PCR Detection System (Cat. No.: 1841000 for C1000™ Thermal Cycler Chassis and Cat. No.: 1845096 for CFX96™ Optical Reaction Module)
- Bio-Rad 96-well PCR Plates, low profile, unskirted, clear (Cat No.: MLL9601) for Bio-Rad CFX96 Real-Time PCR Detection System.
- MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL) (Ref No.: 4346906) for Applied Biosystems™ 7500 Fast Real-Time PCR System.
- Bio-Rad Microseal 'B' PCR Plate Sealing Film, adhesive, optical (Cat No.: MSB1001).
- Benchtop centrifuge with a rotor for 2 mL reaction tubes.
- Centrifuge capable of 1000 x *g/rcf* with a rotor for microtiter plates, if using 96-well reaction plates.
- Vortex mixer.

NOTE	
i	***Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.***

6 Limitations, Warnings and Precautions

The MiRXES Fortitude SARS-CoV-2 & FluA/B Test described here has not been systematically verified for platforms or chemicals other than those mentioned in these instructions.

Read the Instructions for Use carefully before using the product.

NOTE	
i	***This test has been validated but FDA's independent review of this validation is pending. Please include the statement below, in the test report(s) generated:*** ***"the test has been validated but FDA's independent review of this validation is pending"***

6.1 Acceptable Specimens

Nasopharyngeal/oropharyngeal swabs, nasopharyngeal wash/aspirate, nasal wash/aspirate and anterior/mid-turbinate nasal swabs from patients suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider.

6.2 Biosafety Precautions On Specimen Handling

- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Put on protective disposable powder-free gloves, a laboratory coat and eye protection when handling clinical specimens.
- Perform all manipulations of potentially infectious virus specimens within a Class II (or higher) biological safety cabinet (BSC).
- Discard sample and assay waste according to your local safety regulations.

6.3 Handling the MiRXES Fortitude SARS-CoV-2 & FluA/B Test

- Before initial use, check the product and its components for:
  - Cold state upon arrival
  - Integrity
  - Completeness with respect to number, type and filling
  - Correct labelling
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time RT-PCR.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the Kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.

- Always wear protective disposable powder-free gloves when handling kit components.
- The workflow in the laboratory should proceed in a unidirectional manner. Use separated and segregated working areas for:

  (i) sample preparation,

  (ii) reaction setup and

  (iii) amplification/detection activities.
- Always wear disposable powder-free gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separately from all other components of the Kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Do not autoclave reaction tubes after the RT-PCR, since this will not degrade the amplified nucleic acid and risks contaminating the laboratory area.
- Do not use expired components, refer to the label for expiration date.

7 Procedure

7.1 RNA Extraction from Clinical Sample

- Extracted RNA is the starting material for the MiRXES Fortitude SARS-CoV-2 & FluA/B Test.
- MiRXES Fortitude SARS-CoV-2 & FluA/B Test has been validated using RNA samples extracted from clinical samples via QIAamp Viral RNA Mini Kit using sample input of *200 µL* and elution volume of *60 µL* following the extraction procedure provided in manufacturer's instruction.

  Note: Specimens to be added into 2 mL polypropylene microcentrifuge tubes (DNase/RNase free). 800 µL of Buffer-AVL-carrier RNA and 800 µL of ethanol (96-100 %) will be used due to the increased sample volume (200 µL). For the volume of other reagent and detailed procedure please refer to Manufacturer's instruction.
- The Negative Control should be extracted alongside each batch of clinical specimens with input of *200 µL* and elution volume of *60 µL*. Negative Control is used to determine if there has been contamination during RNA extraction and/or plate setup.
- The suitability of the other nucleic acid extraction procedure for use with MiRXES Fortitude SARS-CoV-2 & FluA/B Test should be validated by the user.
- The quality of the extracted RNA has a significant impact on the performance of downstream RT-PCR assays. Ensure that the system used for nucleic acid extraction is compatible with real-time RT-PCR technology.
- A negative result from the MiRXES Fortitude SARS-CoV-2 & FluA/B Test may be due to failed RNA extraction. Interpretation of assay results should take into consideration quality of both clinical sample and extracted RNA.

CAUTION

- If your RNA extraction system uses wash buffers containing ethanol, make sure to eliminate any traces of ethanol prior to elution of the nucleic acid. Ethanol is a strong inhibitor of real-time RT-PCR.
- Maintain the integrity of the sample chain of custody (clinical sample to RNA and RNA to assay) to ensure the patient sample ID is associated with the correct test result.

7.2 Positive Control Preparation

- Positive Control for the MiRXES Fortitude SARS-CoV-2 & FluA/B Test consists of synthetic RNA of the SARS-CoV-2 genome, Influenza A genome, Influenza B genome and Human RNaseP gene segment. It is provided at high concentration and require a dilution before adding into the reaction:
  - Completely thaw the Positive Control vial on ice. Mix the vial using a vortex and briefly spin down the Positive Control.

- Add 198 µL of Nuclease free water into a new microtube. Add 2 µL of Positive Control into the microtube and vortex well.
- Briefly spin down the diluted Positive Control and keep on ice until use.

- The diluted Positive Control does not participate in the extraction process but should be treated as an extracted sample and directly added into the PCR reaction solution.

NOTE: ***Positive Control is to be prepared freshly before run. Do NOT re-freeze diluted Positive Control.***

7.3 RT-PCR Reaction Master Mix Preparation

CAUTION	
	• Do not combine components of assay kits with different lot numbers. • Keep all RNA samples and MiRXES Fortitude SARS-CoV-2 & FluA/B Test components on ice. • Set up all reactions on cold blocks or on ice to minimize RNA degradation. • Briefly spin down all reagents before opening the cap. • Set up the reaction in designated PCR workstation to avoid contamination from other PCR activities.

- Completely thaw all kit components on ice.
- Determine the number of reactions to be prepared per run. Each run must include at least one well of diluted Positive Control and one well of Negative Control eluate.
- Calculate the amount of each component of Master Mix to be added by multiplying the number of reactions (samples plus controls and extra 2 to 4 wells) by the volume per reaction indicated in Table 2.

NOTE: ***It is necessary to make excess reaction master mix to allow for pipetting error.***

- Prepare a sterile, nuclease-free, 1.5 mL tube or 5 mL tube for the Fortitude SARS-CoV-2 & FluA/B Test master mix depending on the volume.
- Dispense the components, by pipetting, in the order of sequence shown in Table 2.
- Mix the master mix thoroughly by gentle pipetting or vortexing. Centrifuge briefly before use.

Table 2. Master Mix Preparation & PCR Reaction Set-up

Sequence	Component Label		Master Mix	Volume per Reaction (µL)
1	1	(INT0076)	Nuclease Free Water	6.0
2	4	(INT0073)	Probes Reaction Mix	12.5
3	6	(INT0075)	Primers - Probes Mix	1.0
4	5	(INT0074)	Reverse Transcriptase	0.5
			Master Mix Volume per Reaction	**20.0**
--	--		**Test Sample** - Extracted RNA from clinical sample or - *Diluted* Positive Control or - Negative Control eluate	5.0
			Total PCR Reaction Volume	**25.0**

7.4 Reaction Setup

CAUTION

- Keep all RNA and reaction Master Mix on ice.
- Place 96-well PCR plate or reaction tube on cool block or on ice during reaction set up to minimize RNA degradation.
- Briefly spin down RNA samples and Master Mix before opening the cap.
- Set up the reaction in designated PCR workstation to avoid contamination from other PCR activities.

- Dispense 20.0 µL of the Master Mix into all sample and control wells of an appropriate optical 96-well reaction plate (See Section 5: Material and Devices Required But Not Provided).
- Add 5.0 µL of extracted RNA samples (eluate from the nucleic acid extraction) or 5.0 µL of the controls (diluted Positive Control or Negative Control eluate) into their designated wells.
- Thoroughly mix the RNA samples or Controls with the Master Mix by gently pipetting up and down.
- Make sure at least one Positive Control and one Negative Control are included in each run.
- Seal the 96-well reaction plate with optical adhesive film (See Section 5: Material and Devices Required But Not Provided).
- Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor for 30 seconds at approximately 1000 x *g*.

7.5 Real-Time PCR Instrument

MiRXES Fortitude SARS-CoV-2 & FluA/B Test was developed and validated to be used with the following instruments:

- Applied Biosystems™ 7500 Fast Real-Time PCR System
- Bio-Rad CFX96 Real-Time PCR Detection System

CAUTION	
	• MiRXES Fortitude SARS-CoV-2 & FluA/B Test may not deliver the same results if used with other systems. • User should perform in-house verification and validation should other PCR system be used.

7.6 Temperature Profile and Cycling

Step	Temperature (°C)	Duration (Hr:Min:Sec)	No. of Cycles	Detection
Reverse Transcription	48	00:15:00	1	-
RT Inactivation / Initial Denaturation	95	00:02:30	1	-
Denaturation	95	00:00:10	45	-
Annealing [Data Collection]	61	00:00:42		√ All Channels

7.7 Fluorescence Detectors (Dyes)

S/N	Target	Channel
1	SARS-CoV-2 (SC2)	FAM
2	Influenza A (Flu A)	Hex[a] / VIC[b]
3	Influenza B (Flu B)	Texas Red
4	Human RNaseP region (Rp)	Cy5

a. Select HEX channel for Flu A on Bio-Rad CFX96 Real-Time PCR System

b. Select VIC channel for Flu A on Applied Biosystems™ 7500 Fast Real-Time PCR System

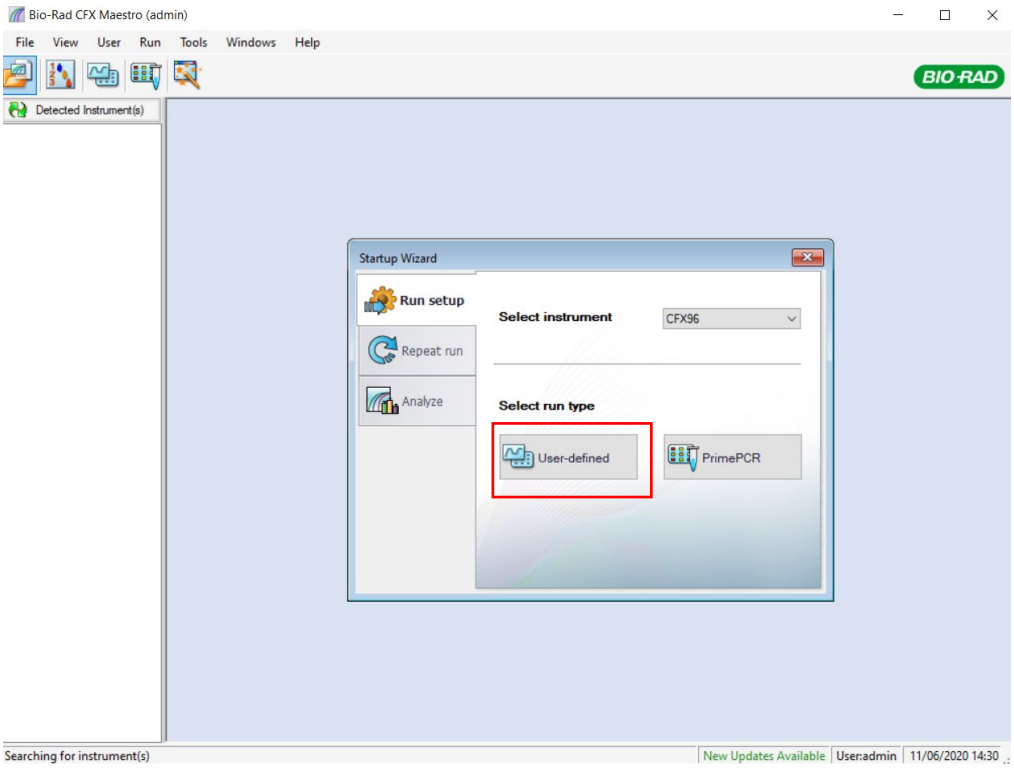
7.8 Programming the Real-Time PCR Instrument

7.8.1 Setup for Bio-Rad CFX96 Real-Time PCR Detection System using Bio-Rad

CFX Maestro

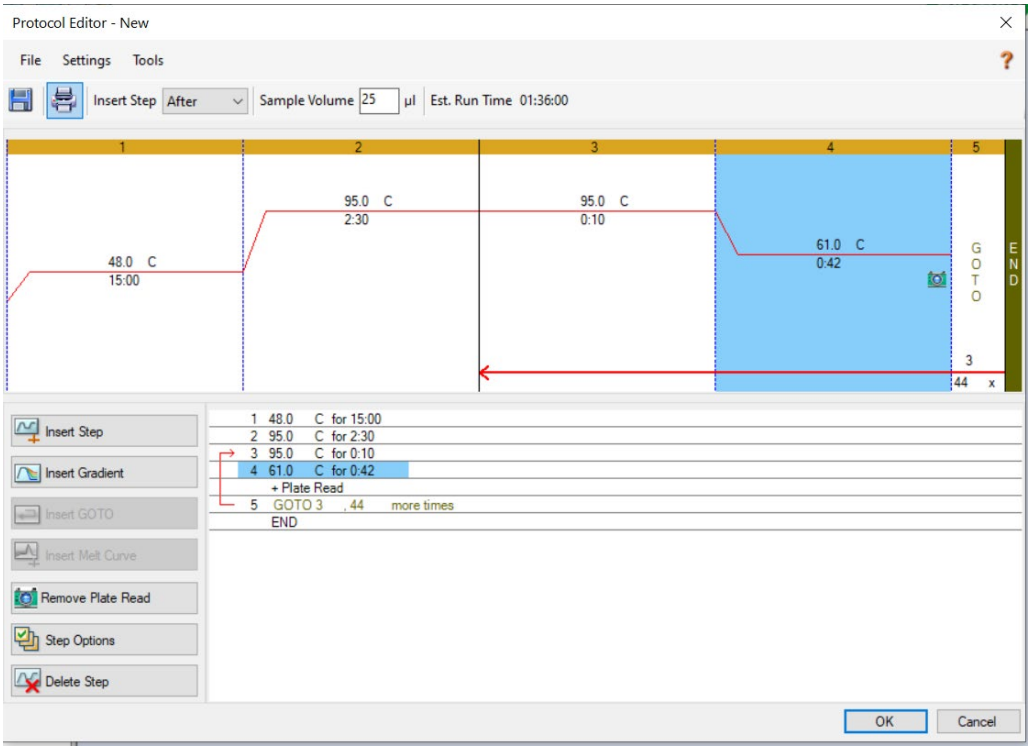
a) Setup a new run by clicking **User-defined** as run type. Refer to Figure 1.

Figure 1: Run Setup



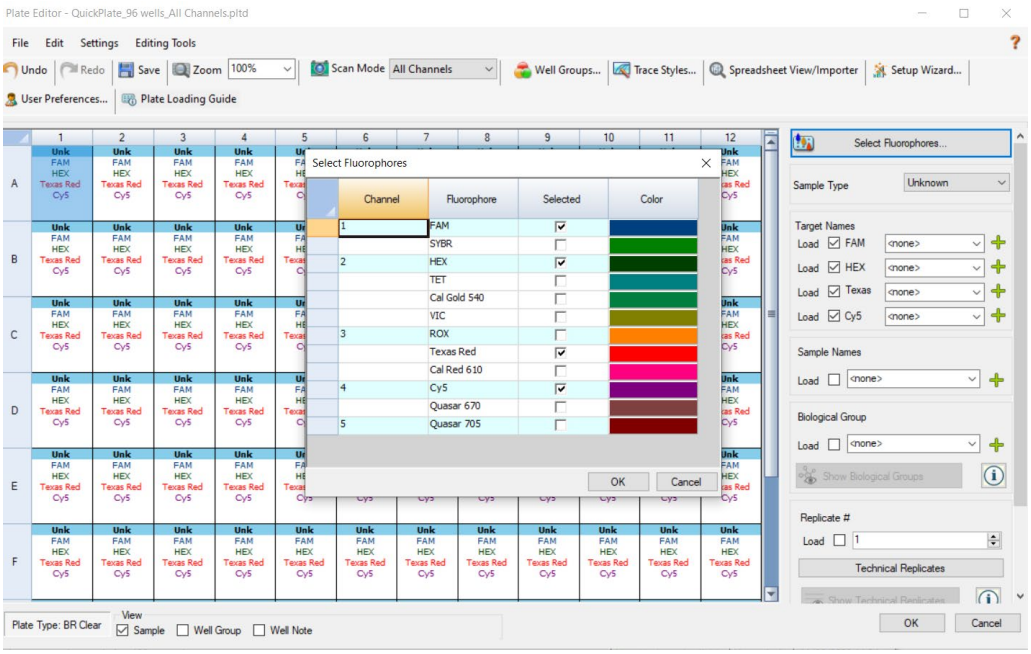
b) Under **Protocol Editor**, click on **Create New** and set protocol according to section 7.6. Input **Sample volume** as '25' µL. Click **OK**. Refer to Figure 2.

Figure 2: Protocol Editor



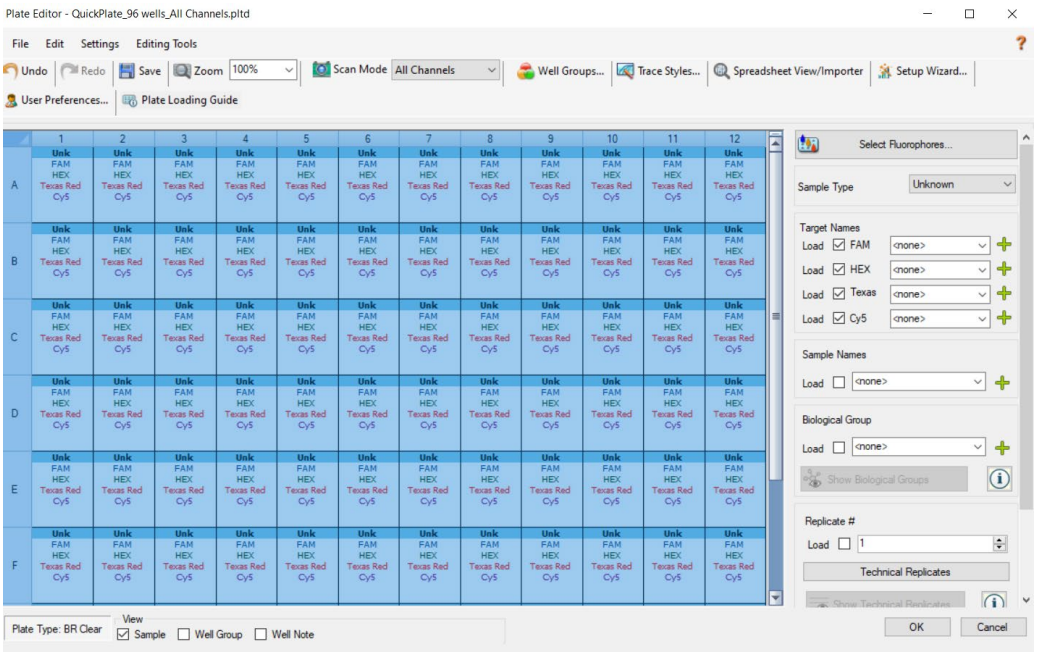
c) Under **Plate Editor**, click on the **Edit selected** to edit. Click on **Select Fluorophores** to select desired fluorophores **FAM**, **HEX**, **Texas Red** and **Cy5**, click **OK**. Refer to Figure 3.

Figure 3: Plate Editor



d) Input Sample Names and Replicates (if applicable). Load all fluorophores to the desired well, click **OK**. Refer to Figure 4.

Figure 4: Load fluorophores

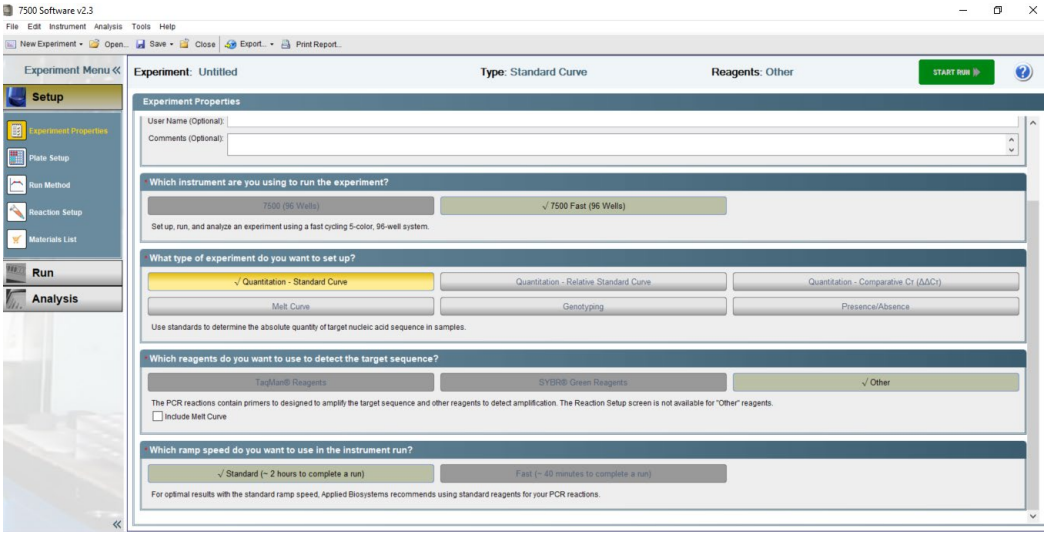


e) Start run.

7.8.2 Setup for Applied Biosystems™ 7500 Fast Real-Time PCR System using 7500 Software v2.3

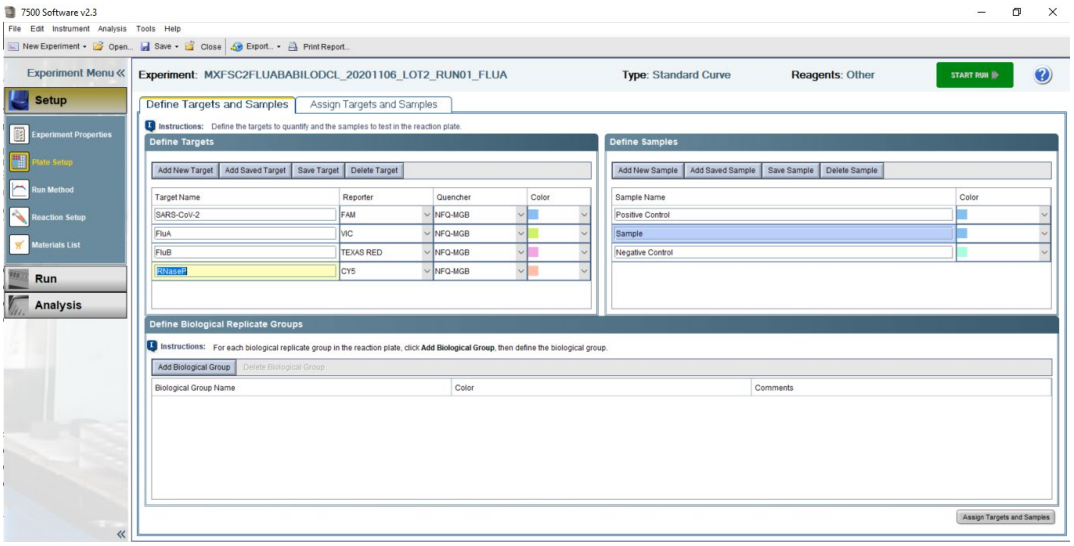
a) Under **Setup/Experiment Properties**, adjust the setup according to Figure 5.

Figure 5: Experiment Properties



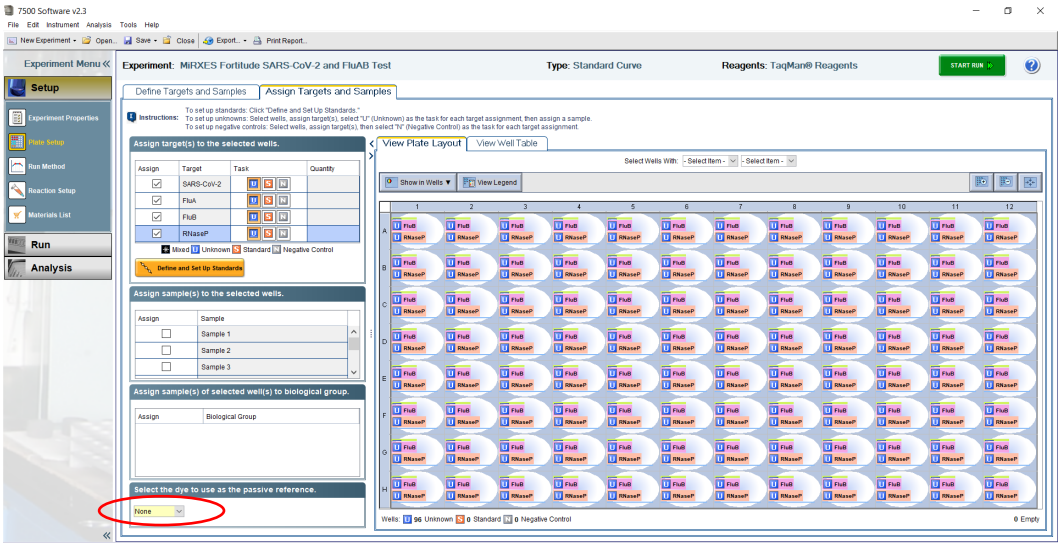
b) Under **Setup/Plate Setup/Define Targets and Samples**, enter **Target Name** and **Reporter** according to Section 7.7. Select **NFQ-MGB** for **Quencher**. Define sample on the RIGHT panel according to experiment layout. Refer to Figure 6.

Figure 6: Plate Setup



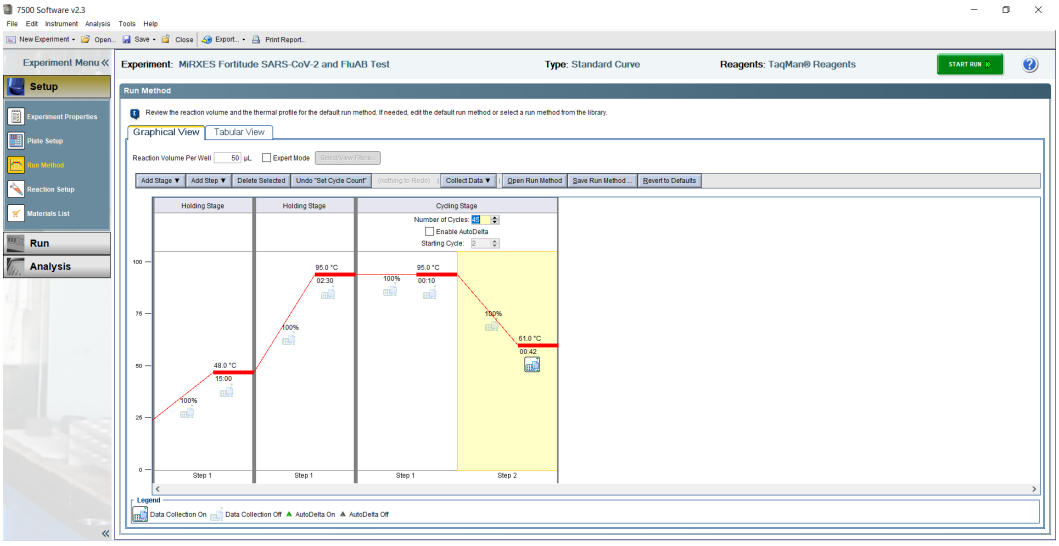
c) Under **Setup/Plate Setup/Assign Targets and Samples**, Assign all the Target and samples to the desired wells and select **None** as **passive reference** at the bottom of the panel. Refer to Figure 7.

Figure 7: Assign Targets and Samples



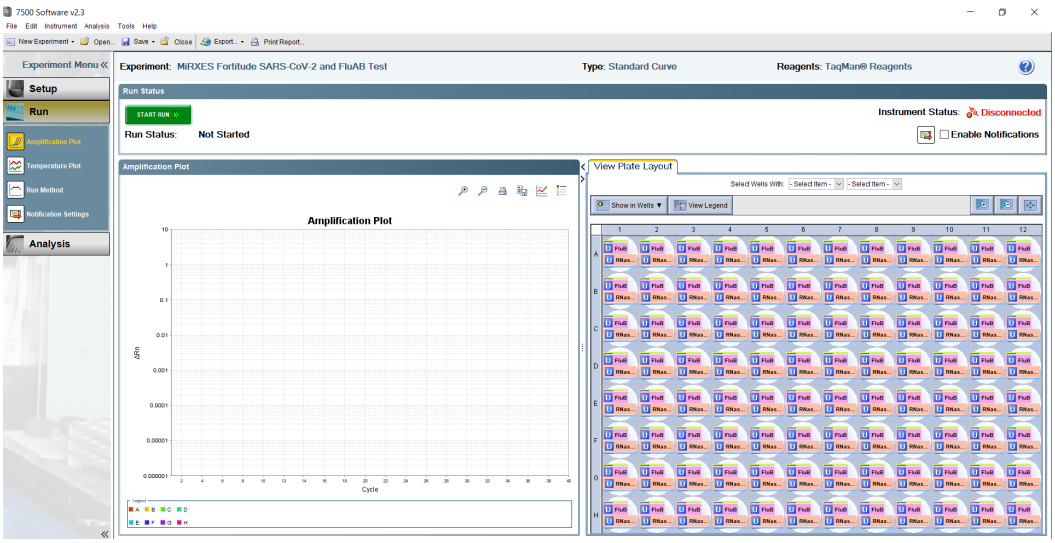
d) Set **Run method** according to section 7.6. Input **Reaction Volume** as '25' µL. Refer to Figure 8.

Figure 8: Run method



e) Start the Run by clicking on the **START RUN**.

Figure 9: Start RUN



8 Data Analysis

- For basic information regarding data analysis on specific real-time PCR instruments, please refer to the user manual of the respective instrument.

CAUTION

- Before making C_q value determination, users MUST inspect individual PCR amplification curve and determine the validity of each PCR reaction (Figure 19).
- All C_q values and data interpretation stated below are based on valid PCR amplification curve.
- C_q values from anomalous PCR amplification profile should be considered invalid.
- Non-sigmoidal amplification curves should be queried before accepting the C_q values. It may be a result of incorrect baseline setting in your instrument's data analysis software or a high level of fluorescent noise during the early cycles of PCR.
- Note that if the plateau phase of the PCR amplification curve is close to the threshold, it indicates weak florescence signal or potential inhibition. Repeat run with freshly extracted RNA samples and/or fresh PCR reagents.

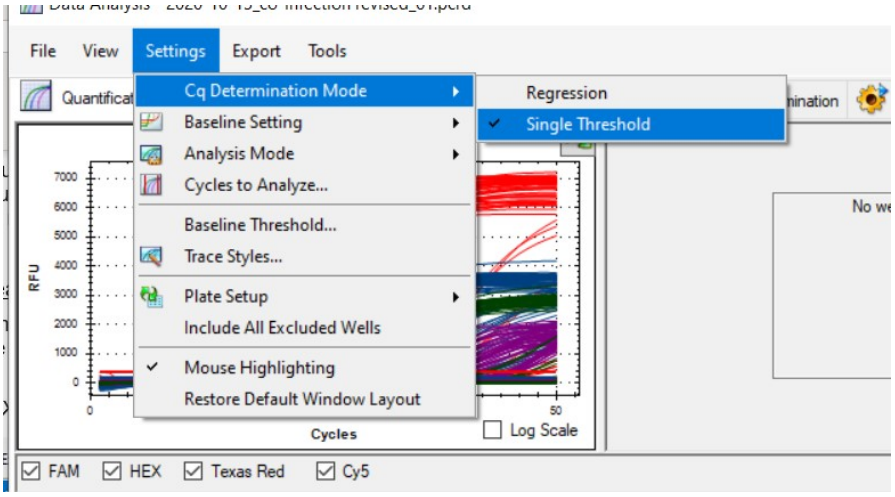
8.1 C_q Determination

Due to potential instrument-to-instrument variation in the fluorescence signal saturation range, instrument calibration might be required to set the appropriate threshold for individual real-time PCR systems.

8.1.1. Bio-Rad CFX96 Real-Time PCR Systems

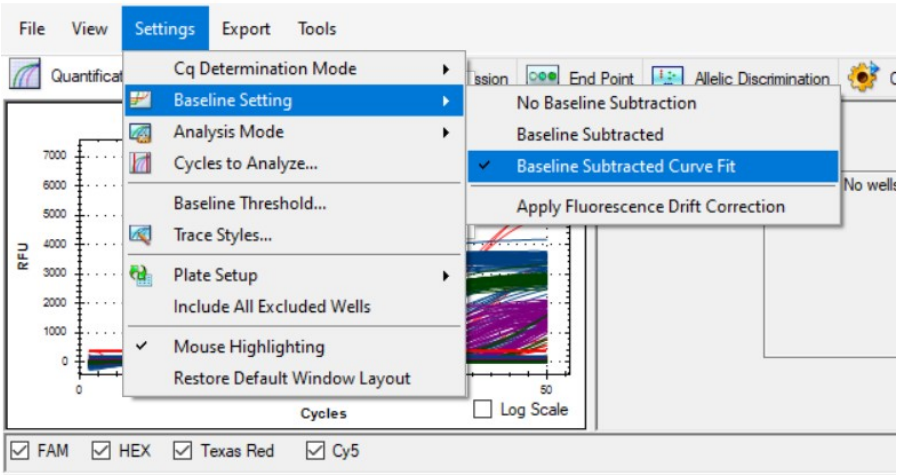
a) After the run has completed select the **Settings** tab on the top panel of the software (See Figure 10). Under **Cq Determination Mode**, select **Single Threshold**.

Figure 10: Bio-Rad CFX96 Real-Time PCR Systems setting



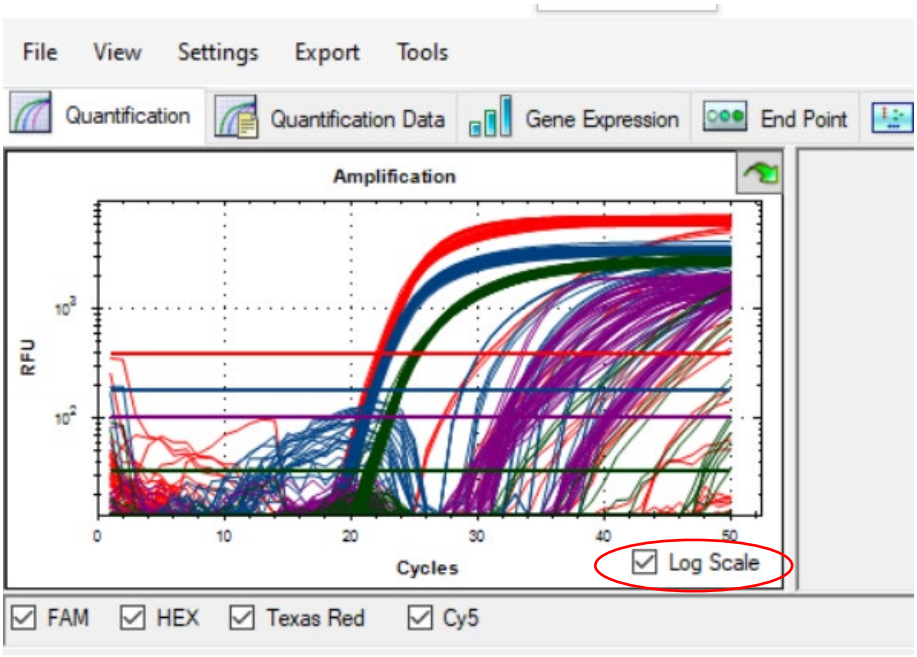
b) Under the **Baseline setting**, select **Baseline Subtracted Curve Fit** (See Figure 11).

Figure 11: Baseline setting



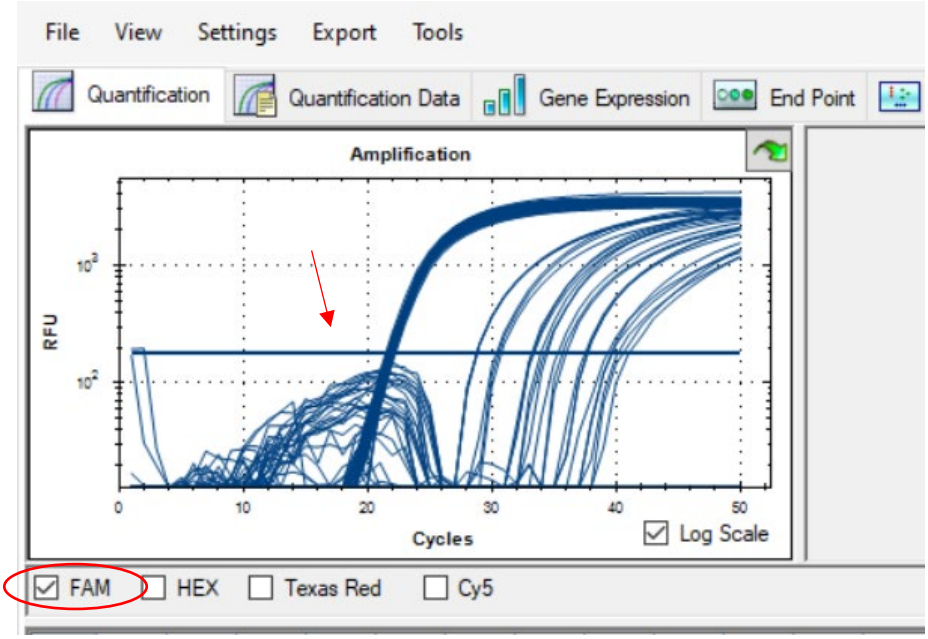
c) Update the graph to Log view by checking the **Log Scale** box located at the left bottom corner of the graph (circled in red in Figure 12).

Figure 12: Log scale



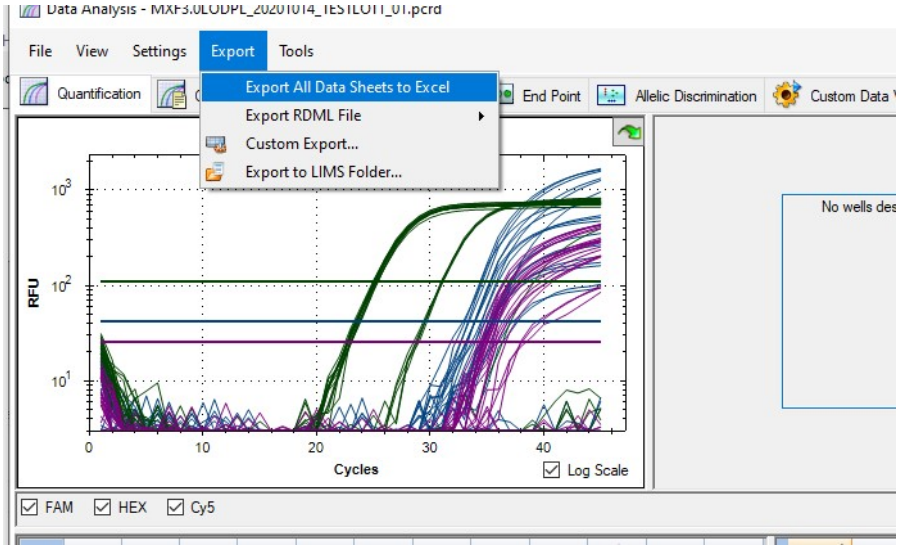
d) Select the **FAM** channel (circled in Red in Figure 13). Drag the threshold line (red arrow) until it lies within the exponential phase of the fluorescence curves and above any background signal (see Figure 13). Repeat this step for each of the channels used (HEX, Texas Red and Cy5).

Figure 13: Setting Threshold



e) Export the Cq values by clicking on the **Export** tab on the top panel and select desired save location for the exported file (See Figure 14).

Figure 14: Export data

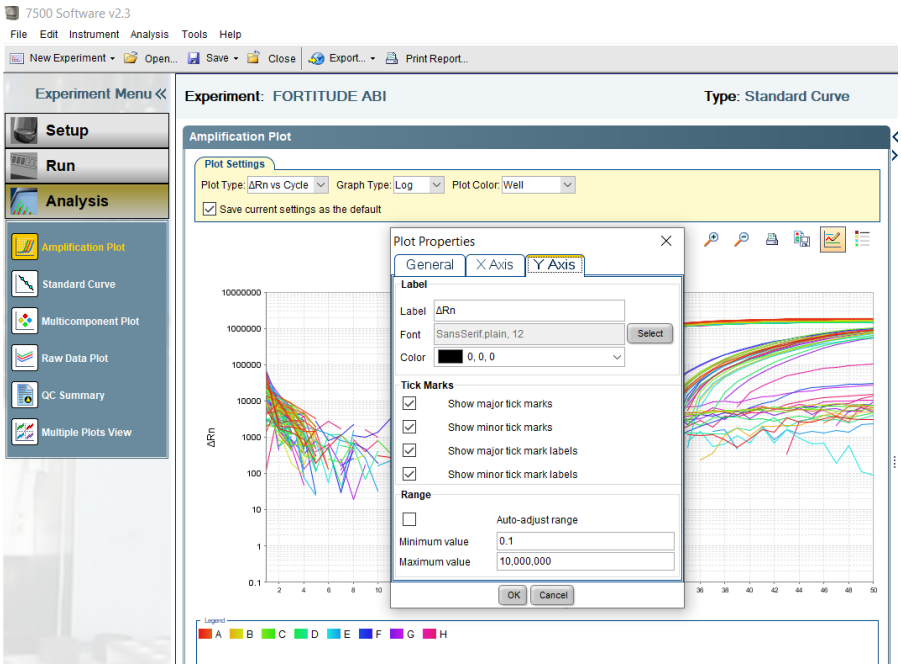


8.1.2. Applied Biosystems™ 7500 Fast Real-Time PCR System

a) After the run has completed, select the **Analysis** tab on the left panel of the software.

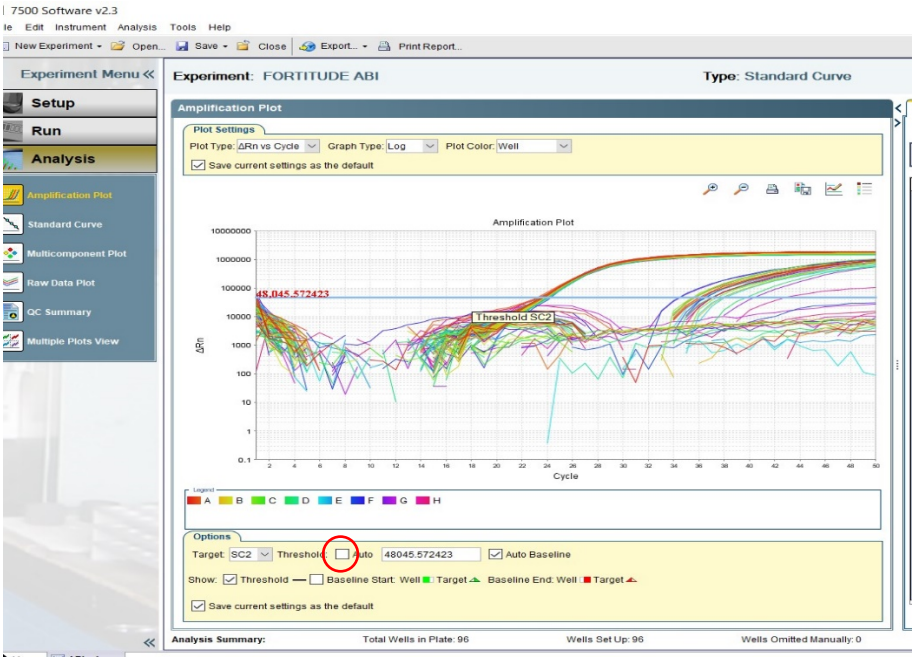
b) Select the **Amplification Plot** tab. Update the graph to **Log** view by right clicking on the Amplification plot. If necessary, adjust the **Y Axis** (see Figure 15).

Figure 15: Log view of Amplification Plot



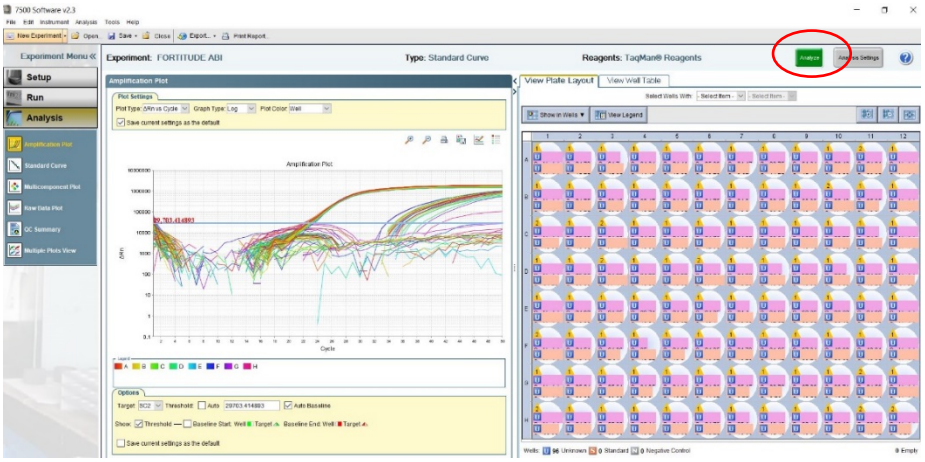
c) Select the FAM channel. Uncheck the **Auto** box in **Options** table (circled in red in Figure 16). Drag the threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal. Make sure **Auto Baseline** box is checked. Repeat this step for each of the channels used (VIC, Texas Red and Cy5).

Figure 16: Threshold Settings



d) Click the **Analyze** button located at the right upper corner (see Figure 17).

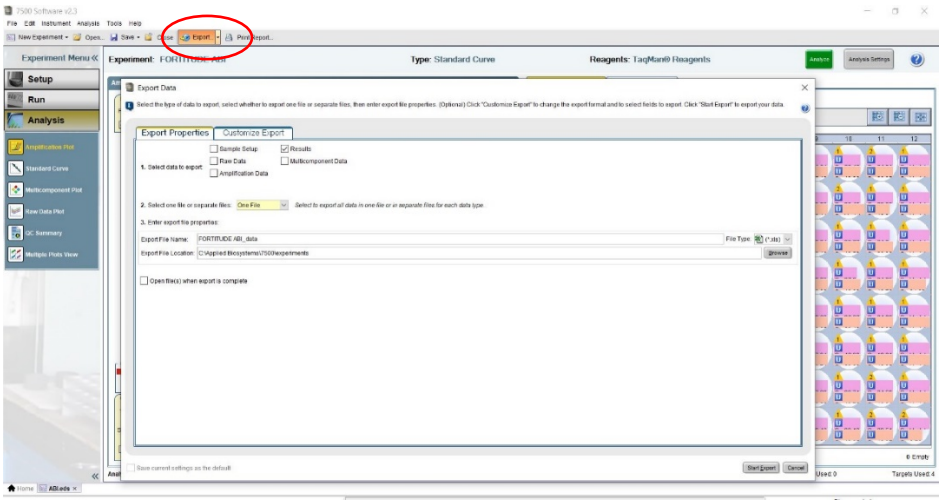
Figure 17: Analyze



e) Save analysis file by selecting File then **Save As** from the main menu.

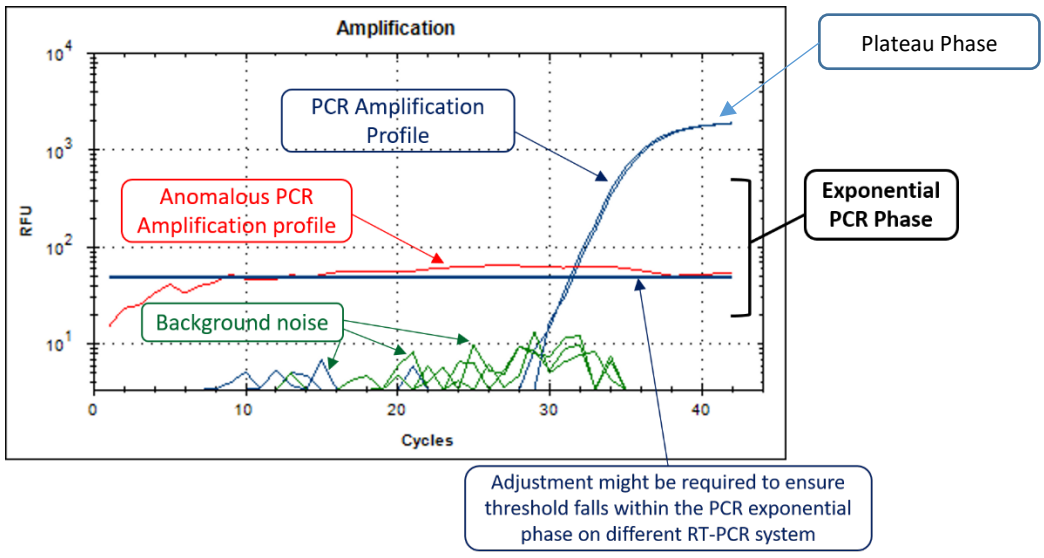
f) Export the Cq values by clicking on the **Export** tab and select desired save location for the exported file (see Figure 18).

Figure 18: Export data



Interpretation of MiRXES Fortitude SARS-CoV-2 & FluA/B Test results MUST take into consideration **the C_q values,** as well as **the shape of the amplification curve**, as shown in Figure 19 below.

Figure 19. Amplification Plot Window



Note:

(1) Anomalous PCR amplification profile in red is signal due to non-specific amplification. C_q values obtained from such amplification curves are not valid and should not be taken into consideration in data analysis and result interpretation. (2) PCR amplification profile in blue shows examples of fluorescence growth curves as a result of specific amplification. Only C_q values obtained from such amplification curves are valid and should be taken into consideration in data analysis and result interpretation.

8.2 Positive and Negative Control Pass Criteria

Table 3: Pass Criteria

Control Type	SC2 (FAM)	Flu A (HEX/Vic)	Flu B (Texas Red)	Rp (Cy5)	To evaluate	Expected Cq
Positive Control (PC)	+	+	+	+	RT-PCR reagent integrity including primers and probes for all targets	SC2 ≤ 40; Flu A ≤ 40; Flu B ≤ 40; Rp ≤ 40;
Negative Control (NC)	-	-	-	-	Contamination of reagents or environmental contamination	SC2: Undetermined; Flu A: Undetermined; Flu B: Undetermined; Rp: Undetermined

Users may perform independent calibration runs on their own instruments to establish a passing criteria. Marked deviation from the recommended or self established passing criteria should be queried before releasing results.

- If the Positive Control does not pass, this indicates a problem with reagent integrity and/or RT-PCR reaction(s). Users should investigate the cause and repeat the run with fresh reagents.
- If the Negative Control is positive for either signals, this indicates a sample contamination may have occurred. Invalidate the run and eliminate the contamination before repeat with a new run.

8.3 Test Sample Interpretation

The cutoff for positive signal **for SC2 is < 42**, **Flu A is < 42**, **Flu B is < 42**, **RP is < 42**.

Table 4: Sample interpretation

C_q Values				**Interpretation**	**Action**
SC2 (FAM)	**Flu A (HEX)**	**Flu B (Texas Red)**	**Rp (Cy5)**		
< 37	≥ 42 or Undetermined	≥ 42 or Undetermined	< 42 or ≥ 42 [a] or Undetermined	SARS-CoV-2 Detected	Report result to sender
37 < C_q < 42	≥ 42 or Undetermined	≥ 42 or Undetermined	< 42 or ≥ 42 [a] or Undetermined	Equivocal [c] for SARS-CoV-2	Consider repeat of extraction and/or RT-PCR or collecting a new specimen [c]
≥ 42 or Undetermined	< 42	≥ 42 or Undetermined	< 42 or ≥ 42 [a] or Undetermined	Flu A detected	Report result to sender
≥ 42 or Undetermined	≥ 42 or Undetermined	< 42	< 42 or ≥ 42 [a] or Undetermined	Flu B detected	Report result to sender
< 42	< 42	≥ 42 or Undetermined	< 42 or ≥ 42 [a] or Undetermined	SARS-CoV-2 and Flu A detected	Report result to sender
< 42	≥ 42 or Undetermined	< 42	< 42 or ≥ 42 [a] or Undetermined	SARS-CoV-2 and Flu B detected	Report result to sender
≥ 42 or Undetermined	< 42	< 42	< 42 or ≥ 42 [a] or Undetermined	Flu A and Flu B detected	Report result to sender
< 42	< 42	< 42	< 42 or ≥ 42 [a] or Undetermined	SARS-CoV-2, Flu A and Flu B detected	Report result to sender
≥ 42 or Undetermined	≥ 42 or Undetermined	≥ 42 or Undetermined	< 42	Not detected	Report result to sender
≥ 42 or Undetermined	≥ 42 or Undetermined	≥ 42 or Undetermined	≥ 42 or Undetermined	**Invalid** [b] result	Consider repeat of extraction and/or RT-PCR or collecting a new specimen

a –. With positive viral signals, it is acceptable for Human RNase P (Cy5) to not show valid amplification because of substrate competition with viral genes, especially at high viral load.

b – The Human RNase P (Rp) in the MiRXES Fortitude SARS-CoV-2 & FluA/B Test serves as an internal control for the assay that is used in conjunction with the data from other targets for interpretation of an individual specimen. Rp should be positive (< 42 Ct) for all clinical specimens in the absence of signal for any of the viral targets. If Rp is negative in the presence of positive result for any of the viral targets, the

viral target result should be considered valid. However if all viral targets generate negative results and Rp is also negative, the test is considered invalid. Failure to detect Rp in clinical specimen could indicate:

1) *Insufficient nucleic acid quantity from RNA extraction*
2) *Poor specimen quality or loss of specimen integrity*
3) *Improper assay execution*
4) *Reagent or equipment malfunction.*

Repeat RT-PCR/nucleic acid extraction for the specimen. Collection of a new specimen for repeat testing should be considered.

*c – In case of **Equivocal** result(s), the user may consider the following options*

1) *Re-extract RNA and repeat the test with MiRXES Fortitude SARS-CoV-2 & FluA/B Test, with strict adherence to the procedure guidelines.*
2) *Report as 'Equivocal', with a comment suggesting re-sampling of patient if clinically indicated.*

9 Performance Evaluation

Performance of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was evaluated by both Analytical Validation (Analytical Sensitivity, Inclusivity, Analytical Specificity, Co-infection LoD, Interfering Substances and Clinical Specimen Stability) and Clinical Validation.

9.1 Analytical Sensitivity

Analytical sensitivity of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was determined in limit of detection (LoD) using inactivated viral stocks of an Influenza A virus, an Influenza B virus and a SARS-CoV-2 virus (Table 5) to determine the lowest concentration of each virus at which 95% of all replicates are positive.

Table 5: Viral stocks

Virus	Product	Vendor/Catalogue Number
Influenza A	Influenza A virus (H3N2), Purified	ATCC® VR-1882PQ™
Influenza B	Influenza B virus (Yamagata Lineage), Purified	ATCC® VR-1804PQ™
SARS-CoV-2	SARS-Related Coronavirus 2, Isolate USA-WA1/2020, Gamma-Irradiated	BEI Resources NR-52287

a) Range-finding

Range-finding was done to determine an estimated LoD. Serial, 2 fold dilution of each virus were prepared with 0.09% Saline. The diluted samples were spiked into Negative Human Nasopharyngeal Swab samples to make contrived samples. Triplicate samples of each dilution were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. The estimated LoD was defined as the lowest concentration at which each target (SARS-CoV-2, Influenza A, Influenza B) demonstrated 100 % positivity (3 out of 3 replicates). Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Bio-Rad CFX96 Real-Time PCR Systems. Results for range-finding across all three targets are presented in Table 6.

Table 6: Range-finding

Viral Target	Conc. (GE/µL)	Cq			Average Cq	Detection Rate
SARS-CoV-2	2	31.72	31.82	31.86	31.8	100%
	1	32.05	32.6	32.35	32.33	100%
	0.5	33.76	33.39	33.73	33.63	100%
	0.25	33.68	33.46	33.58	33.57	100%
	0.125	UD	UD	UD	N/A	0%
Influenza A	8	32.51	32.24	32.14	32.3	100%
	4	32.03	34.51	34.04	33.53	100%
	2	35.63	35.01	34.8	35.15	100%
	1	35.3	35.75	UD	35.53	67%
	0.5	UD	41.11	37.41	39.26	67%
Influenza B	8	34.01	33.27	35.61	34.3	100%
	4	33.06	30.93	35.06	33.02	100%
	2	36.66	34.6	UD	35.63	67%
	1	UD	UD	36.15	36.15	33%
	0.5	UD	UD	UD	N/A	0%

UD = Undetermined; N/A = Not Applicable

b) Confirmatory LoD

The confirmatory study of the LoD for the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was demonstrated by spiking virus into Negative Human Nasopharyngeal Swab samples. 20 individually extracted samples at and below the estimated LoD were tested for each virus. LoD was determined at the lowest concentration of each virus at which ≥95% (19/20) of all replicates are tested positive. LoD results are summarized in Table 7. LoDs for SARS-CoV-2, Flu A abd Flu B are 0.25 GE/µL, 1 GE/µL and 8 GE/µL, respectively.

Table 7: Limit of Detection for each virus

LoD	Region	Fluor Channel	Concentration (GE/µl)	Detection	Detection Rate
Confirmatory	SARS-CoV-2	FAM	0.25	20/20	100 %
			0.125	18/20	90 %
	Flu A	HEX	2	19/20	95 %
			1	19/20	95 %
			0.5	15/20	75 %
	Flu B	Texas Red	8	20/20	100 %
			4	10/20	50 %
			2	11/20	55 %

c) Bridging LoD on Applied Biosystems™ 7500 Fast Real-Time PCR System

The refined LoD for the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was demonstrated by spiking virus into Negative Human Nasopharyngeal Swab samples to make contrived samples at 0.5x LoD, 1x LoD, 1.5x LoD an 2x LoD defined in Table 9.1 (b). 5 replicate samples of each dilution were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. The refined LoD was defined as the lowest concentration at which each target (SARS-CoV-2, Influenza A, Influenza B) demonstrated 100 % positivity (5 out of 5 replicates). Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Applied Biosystems 7500 Fast Real-Time PCR Systems. The results for refined LoD are summarised in Table 8.

Table 8: Refined LoD

Viral Target	Conc. (LoD)	Cq					Average Cq	Detection Rate
SARS-CoV-2	0.5x LoD	UD	39.98	40.03	39.09	39.86	39.74	80%
	1x LoD	36.56	36.74	38.11	36.35	38.01	37.15	100%
	1.5x LoD	37.51	40.43	36.30	35.38	35.28	36.98	100%
	2x LoD	36.66	36.91	36.38	35.25	36.81	36.41	100%
Influenza A	0.5x LoD	UD	41.82	UD	42.29	UD	42.06	40%
	1x LoD	39.39	39.36	38.74	41.93	39.31	39.75	100%
	1.5x LoD	38.07	37.69	39.89	40.42	38.58	38.93	100%
	2x LoD	37.71	37.63	35.00	36.65	37.85	36.97	100%
Influenza B	0.5x LoD	41.39	41.90	37.25	36.98	33.68	38.24	100%
	1x LoD	42.82	31.84	38.64	38.52	37.92	37.95	100%
	1.5x LoD	34.01	36.28	30.19	37.13	37.98	35.12	100%
	2x LoD	38.02	36.98	37.63	34.86	39.56	37.41	100%

UD=Undetermined

The confirmatory study of the LoD for the MiRXES Fortitude SARS-CoV-2 & FluA/B Test on Applied Biosystems™ 7500 Fast Real-Time PCR System was demonstrated by spiking virus into Negative Human Nasopharyngeal Swab samples. 20 individually extracted samples at and below the refined LoD were tested for each virus. LoD was determined at the lowest concentration of each virus at which ≥95% (19/20) of all replicates are tested positive. LoD results on Applied Biosystems™ 7500 Fast Real-Time PCR System are summarized in Table 9.

Table 9: Limit of Detection for each virus on Applied Biosystems™ 7500 Fast Real-Time PCR System

Viral Target	LoD (x)	LoD (GE/µL)
SARS-CoV-2	1x LoD	0.25
Influenza A	1x LoD	1
Influenza B	1x LoD	8

9.2 Inclusivity

In Silico

SARS-CoV-2 Primers-Probes

In silico analysis was performed to determine the inclusivity between the SARS-CoV-2 primers/probes from the MiRXES Fortitude SARS-CoV-2 & FluA/B Test and a total of 408,035 present SARS-CoV-2 sequences available in the GISAID database as of 25th January 2021. Of these, 24160 contain the defining mutations of VOC202012/01 (commonly known as lineage B.1.1.7 or UK variant) and 581 sequences correspond to 501.V2 (commonly known as B.1.351 lineage or South African variant). All sequences were aligned using MAFFT and the corresponding primer(probe)s region of each viral sequence to identify the presence of any mismatches/insertion/deletions.

SARS-CoV 2 Target (Total Number of Sequences = 384459)			Mismatches		
	Sequences	Percentage (%)	Forward	Reverse	Probe
ORF	381396	99.2	0	0	0
	480	0.1	≥1	0	0
	1138	0.3	0	≥1	0
	1430	0.4	0	0	≥1
	11	0.0	0	≥1	≥1
	2	0.0	≥1	0	≥1
	2	0.0	≥1	≥1	0
	0	0.0	≥1	≥1	≥1

SARS-CoV 2 Target (Total Number of Sequences = 384918)			Mismatches		
	Sequences	Percentage (%)	Forward	Reverse	Probe
N	380993	99.0	0	0	0
	1599	0.4	≥1	0	0
	747	0.2	0	≥1	0
	1568	0.4	0	0	≥1
	1	0.0	0	≥1	≥1
	6	0.0	≥1	0	≥1
	4	0.0	≥1	≥1	0
	0	0.0	≥1	≥1	≥1

Overall >98.2 % of aligned sequences have 100% homology to both of the primer (probe)s sequence of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test. None of the VOC202012/01 sequences or 501.V2 sequences have mismatch in either ORF or N

primer set from the MiRXES SARS-CoV-2 & FluA/B Test. There are 81 sequences from the GSAID database possess mismatches in both ORF and N primer sets.

It has shown that single internal mismatches do not have a significant effect on the PCR product yield, but those at the 3'-terminal base have varied effects. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches being at the 3'-terminal, showed a great reduction in PCR product yield[1]. For the 81 affected sequences, the mismatch on their respective primer/probe is at most 1 nucleotide hence reactivity of the specific oligonucleotides included in the MiRXES Fortitude SARS-CoV-2 & FluA/B Test is not expected to be affected.

Influenza A primers-probe

In silico analysis was performed to determine the inclusivity between the Influenza A primers/probe from the MiRXES Fortitude SARS-CoV-2 & FluA/B Test and a total of 9513 present Flu A (2019-07-01 to 2020-09-17) sequences available in the GISAID database as of 17th September 2020. These sequences were aligned using MAFFT with the corresponding primer(probe) region of each viral sequence to identify the presence of any mismatches/insertion/deletions.

Flu A Target (Total Number of Sequences = 9491)		Mismatches		
Sequences	**Percentage (%)**	**Forward**	**Reverse**	**Probe**
8913	93.9	0	0	0
373	3.9	≥ 1	0	0
158	1.7	0	≥ 1	0
36	0.4	0	0	≥ 1
1	0.0	0	≥ 1	≥ 1
0	0.0	≥ 1	0	≥ 1
10	0.1	≥ 1	≥ 1	0
0	0.0	≥ 1	≥ 1	≥ 1

Overall > 93.9% of aligned sequences have 100% homology to the primer (probe)s sequence of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test.

There are 27 sequences with 2 mismatches. (10 sequences with 1 mismatch in forward primer and 1 mismatch in reverse primer; 16 sequences with 2 mismatches in reverse primer; 1 sequence with 1 mismatch in reverse primer and 1 mismatch in probe). No sequences had more than 2 mismatches.

It has shown that single internal mismatches does not have a significant effect on the PCR product yield, but those at the 3'-terminal base had varied effects. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches being at the 3'-terminal, showed a great reduction in PCR product yield[1]. For the affected sequence the mismatch on their respective primer/probe is only 1 or 2 nucleotides located at least 5 nucleotides from the 3'-terminal, hence reactivity of the specific oligonucleotides included in the MiRXES Fortitude SARS-CoV-2 & FluA/B Test is not expected to be affected.

Influenza B primers/probe

In silico analysis was performed to determine the inclusivity between the Influenza B primers/probe from the MiRXES Fortitude SARS-CoV-2 & FluA/B Test and a total of 4734 present Flu B (2019-07-01 to 2020-09-17) sequences available in the GISAID database as of 17th September 2020. These sequences were aligned using MAFFT with the corresponding primer(probe) region of each viral sequence to identify the presence of any mismatches/insertion/deletions.

Flu B Target (Total Number of Sequences = 4722)		Mismatches		
Sequences	**Percentage (%)**	**Forward**	**Reverse**	**Probe**
4270	90.4	0	0	0
6	0.1	≥ 1	0	0
317	6.7	0	≥ 1	0
113	2.4	0	0	≥ 1
4	0.1	0	≥ 1	≥ 1
12	0.3	≥ 1	0	≥ 1
0	0.0	≥ 1	≥ 1	0
0	0.0	≥ 1	≥ 1	≥ 1

Overall > 90.4% of aligned sequences have 100% homology to the primer (probe)s sequence of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test.

There are 42 sequences with 2 mismatches.(11 sequences with 1 mismatch in forward primer and 1 mismatch in probe; 3 sequences with 1 mismatch in reverse primer and 1 mismatch in probe; 28 sequences with 2 mismatches in reverse primer; 3 sequences have 3 mismatches). No sequences had more than 3 mismatches.

It has shown that single internal mismatches does not have a significant effect on the PCR product yield, but those at the 3'-terminal base had varied effects. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches being at the 3'-terminal, showed a great reduction in PCR product yield[1]. For the affected sequence the mismatch on their respective primer/probe is only 1 or 2 nucleotides located at least 5 nucleotides from the 3'-terminal, hence reactivity of the specific oligonucleotides included in the MiRXES Fortitude SARS-CoV-2 & FluA/B Test is not expected to be affected.

[1] Kwok, S. et al (1990) *Effects of primer – template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies*, Nuclei Acids Research, Vol. 18, No. 4

Wet lab

The inclusivity of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was evaluated using viruses or genomic RNA from 11 Influenza A, 4 Influenza B and 1 SARS-CoV-2. The genomic RNAs were prepared at 41.67 copies/reaction whilst the viruses were prepared at around 3x LoD in Negative Human Nasopharyngeal Swab samples. Triplicate samples of each were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Bio-Rad CFX96 Real-Time PCR Systems. The MiRXES Fortitude SARS-CoV-2 & FluA/B Test was able to detect all the strains listed in Table 10.

Table 10: Inclusivity

No.	Strain	Source	Catalog No.
1	A/Brisbane/10/2007 (H3N2)	BEI	NR-20081
2	A/Brisbane/59/2007 (H1N1)	BEI	NR-20080
3	A/Wisconsin/67/2005 (HA, NA) x A/Puerto Rico/8/1934 (H3N2), Reassortant X-161B	BEI	NR-10045
4	Kilbourne F11: A/Rockefeller Institute/5/1957 (H2N2) Influenza A virus	BEI	NR-9678
5	Kilbourne F63: A/NWS/1934 (HA) x A/Rockefeller Institute/5/1957 (NA) (H1N2), Reassortant NWS-F Influenza A virus	BEI	NR-9677
6	Kilbourne F181: A/duck/Singapore/645/1997 (H5N3), Wild Type Influenza A virus	BEI	NR-9682
7	A/New York/392/2004(H3N2)	Twist	Control (103002)
8	A/California/07/2009(H1N1)	Twist	Control (103001)
9	A/Singapore/G2-25.1/2014 (H1N1) virus	NUS	N/A
10	A/Singapore/CDC-204/2014 (H3N2) virus	NUS	N/A
11	A/Singapore/Aichi/2/1968 (H3N2) virus	NUS	N/A
12	B/Nevada/03/2011 (Victoria Lineage)	BEI	NR-45848
13	B/Ohio/01/2005 (Victoria Lineage)	BEI	NR-43753
14	B/Lee/1940	Twist	Control (103003)
15	B/Singapore/G2-14.1/2014 (Victoria lineage) virus	NUS	N/A
16	Australia/VIC01/2020	Twist	Control (102019)

9.3 Analytical Specificity / Cross-reactivity

In silico

In silico exclusivity analysis was performed to determine the likely cross-reactivity between all the organisms listed in Table 11 with the MIRXES Fortitude SARS-CoV-2 & FluA/B Test primers and probes. Sequences for each organism were downloaded from NCBI and a blast search on these sequences were ran against the MiRXES Fortitude SARS-CoV-2 & FluA/B Test primers and probes. Based on the criteria that primers and probes must achieve at least 80% homology with any of the sequences from these organisms for detection, none of the organisms listed are potentially cross-reactive.

Table 11: In Silico Cross-reactivity

Organism	Number of Sequences Deposited in NCBI
Influenza C	2260
Adenovirus type 7a	10
Adenovirus type 2	944
Bordetella pertussis	425446
Candida albicans	26280
Chlamydia pneumoniae	9056
Corynebacterium diphtheriae	38350
Cytomegalovirus	8975
Enterovirus (EV68)	107
Epstein Barr Virus	10850
Escherichia coli	8418791
Haemophilus influenzae	112386
Human coronavirus 229E	546
Human coronavirus HKU1	416
Human coronavirus NL63	913
Human coronavirus OC43	1426
Human Metapneumovirus	9585
Herpes simplex virus	9304
Varicella-zoster virus	1153
Lactobacillus acidophilus	5373
Lactobacillus plantarum	114002
Legionella pneumophila	94624
Measles virus	18346
MERS-coronavirus	1438
Moraxella catarrhalis	21915
Mumps Virus	10743
Mycobacterium tuberculosis	1455159
Mycoplasma pneumoniae	10978
Neisseria elongata	1445
Neisseria meningitidis	649491
Parainfluenza virus 1	827
Parainfluenza virus 2	595
Parainfluenza virus 3	2155
Parainfluenza virus 4	11
Parechovirus	5747
Pseudomonas aeruginosa	2358781
Pneumocystis jirovecii	9219
Respiratory Syncytial Virus	25165
Human Rhinovirus	15256
SARS-coronavirus (SARS-CoV-1)	1065
Staphylococcus aureus	2094762
Staphylococcus epidermidis	344713
Streptococcus salivarius	14168
Streptococcus pneumoniae	2126942
Streptococcus pyogenes	145609

Wet Lab

The MiRXES Fortitude SARS-CoV-2 & FluA/B Test was evaluated for cross-reactivity with normal or pathogenic organisms associated with respiratory tract listed in Table 12. Virus particles or Nucleic acids of 34 organisms, and pooled Human Nasopharyngeal Swab samples were being put through extraction in the background of COPAN UTM™ using QIAamp Viral RNA Mini kit in replicates. The results indicated there is no reactivity of the MiRXES SARS-CoV-2 & FluA/B Test in all 34 organisms and pooled Human Nasopharyngeal Swab samples.

Table 12: Wet Lab Cross-reactivity

No.	Pathogen	Item name (Provider)	Testing conc. (Copies/mL)
1	Adenovirus type 7a	Quantitative Genomic DNA from Human adenovirus 7 (ATCC)	> 5 x 10^6 copies/mL
2	Adenovirus type 1	NATtrol Adenovirus Type 1 (Zeptometrics)	unknown
3	Bordetella pertussis	Quantitative Genomic DNA from Bordetella pertussis (ATCC)	> 1 x 10^7 copies/mL
4	Candida albicans	Quantitative Genomic DNA from Candida albicans (ATCC)	> 1 x 10^7 copies/mL
5	Cytomegalovirus	Quantitative Genomic DNA from Human herpesvirus 5 (HHV-5) (ATCC)	> 5 x 10^6 copies/mL
6	Enterovirus (EV68)	Quantitative Genomic RNA from Enterovirus 68 (ATCC)	> 5 x 10^6 copies/mL
7	Haemophilus influenzae	Quantitative Genomic DNA from Haemophilus influenzae (ATCC)	> 1 x 10^7 copies/mL
8	Human coronavirus 229E	NATtrol Coronavirus 229E (Zeptometrics)	unknown
9	Human coronavirus HKU1	Quantitative Synthetic Human coronavirus HKU1 RNA (ATCC)	> 5 x 10^6 copies/mL
10	Human coronavirus NL63	NATtrol Coronavirus NL63 (Zeptometrics)	unknown
11	Human coronavirus OC43	NATtrol Coronavirus OC43 (Zeptometrics)	unknown
12	Human Metapneumovirus	Quantitative Synthetic Human metapneumovirus (hMPV) RNA (ATCC)	> 5 x 10^6 copies/mL
13	Herpes simplex virus	Quantitative Genomic DNA from Human herpesvirus 2 (HSV-2) (ATCC)	> 5 x 10^6 copies/mL
14	Varicella-zoster virus	Quantitative Genomic DNA from Human herpesvirus 3 (HHV-3) (ATCC)	> 2 x 10^7 copies/mL
15	Legionella pneumophila	Quantitative Genomic DNA from Legionella pneumophila subsp. pneumophila (ATCC)	> 1 x 10^7 copies/mL
16	Measles virus	RNA from Measles virus, strain Edmonston (ATCC)	> 5 x 10^6 copies/mL
17	MERS-coronavirus	NATtrol MERS-CoV stock (Zeptometric)	unknown
18	Mumps Virus	Quantitative Genomic RNA from Mumps virus (ATCC)	> 5 x 10^6 copies/mL
19	Mycobacterium tuberculosis	Quantitative Genomic DNA from Mycobacterium tuberculosis (ATCC)	> 1 x 10^7 copies/mL
20	Mycoplasma pneumoniae	Quantitative Genomic DNA from Mycoplasma pneumoniae (ATCC)	> 1 x 10^7 copies/mL
21	Neisseria meningitidis	Quantitative Genomic DNA from Neisseria meningitidis (ATCC)	> 1 x 10^7 copies/mL
22	Parainfluenza virus 1	Quantitative Genomic RNA from Human parainfluenza virus 1 (ATCC)	> 5 x 10^6 copies/mL
23	Parainfluenza virus 2	Quantitative Genomic RNA from Human parainfluenza virus 2 (ATCC)	> 5 x 10^6 copies/mL

No.	Pathogen	Item name (Provider)	Testing conc. (Copies/mL)
24	Parainfluenza virus 3	NATtrol Parainfluenza Type 3 (Zeptometric)	unknown
25	Parainfluenza virus 4	NATtrol Parainfluenza Type 4 (Zeptometric)	unknown
26	Parechovirus	Quantitative Synthetic Human parechovirus 3 RNA (ATCC)	> 5 x 10^6 copies/mL
27	Pseudomonas aeruginosa	Quantitative Genomic DNA from Pseudomonas aeruginosa (ATCC)	> 1 x 10^7 copies/mL
28	Respiratory Syncytial Virus	Quantitative Genomic RNA from Human respiratory syncytial virus (ATCC)	> 5 x 10^6 copies/mL
29	Human Rhinovirus	Quantitative Genomic RNA from Human rhinovirus 16 (ATCC)	> 5 x 10^6 copies/mL
30	SARS-coronavirus (SARS-CoV-1)	NATtrol Coronavirus SARS stock (Zeptometrics)	unknown
31	Staphylococcus aureus	Quantitative Genomic DNA from Staphylococcus aureus subsp. aureus (ATCC)	> 1 x 10^7 copies/mL
32	Staphylococcus epidermidis	Staphylococcus Epidermidis MRSE; RP62A, DNA (Zeptometrics)	> 1 x 10^7 copies/mL
33	Streptococcus pneumoniae	Quantitative Genomic DNA from Streptococcus pneumoniae (ATCC)	> 1 x 10^7 copies/mL
34	Streptococcus pyogenes	Quantitative Genomic DNA from Streptococcus pyogenes (ATCC)	> 1 x 10^7 copies/mL
35	Human Negative Swab	Human Normal Nasopharyngeal Swab (BioIVT)	N/A

9.4 Co-infection LoD study

Co-infection LoD study was performed to assess potential competitive interference between SARS-CoV-2, Influenza A and Influenza B. Samples were tested in conditions where low concentration of any two tagets were mixed with high (1 x 10^4 GE/µL) concentration of the third target. Viruses were spiked into Negative Human Nasopharyngeal Swab samples in different combination (Table 13). Triplicate samples of each combination were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Bio-Rad CFX96 Real-Time PCR Systems. The MiRXES Fortitude SARS-CoV-2 & FluA/B Test was able to detect all three viruses in below combination:

Table 13: Combination of Co-infection Condition

	Combination 1	Combination 2	Combination 3
Influenza A	10 GE/µL	1 x 10^4 GE/µL	10 GE/µL
Influenza B	20 GE/µL	20 GE/µL	1 x 10^4 GE/µL
SARS-CoV-2	1 x 10^4 GE/µL	1.25 GE/µL	1.25 GE/µL

9.5 Interfering Substances Study

Potential interfering substances from respiratory specimens as listed in Table 14 were tested for the ability to generate false negative results. Viruses were spiked into Negative Human Nasopharyngeal Swab samples to make contrived samples at the concentration of 3x LoD. Interfering substances were added into the contrived samples. Triplicate samples of each were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Applied Biosystems™ 7500 Fast Real-Time PCR Systems. The MiRXES Fortitude SARS-CoV-2 & FluA/B Test was able to detect all three viruses in the presence of interfering substances at the concentration listed in Table 14.

Table 14: Interfering substances and their concentration in the samples

No.	Interfering substances	Active Ingredient	Stock Concentration	Concentration in sample (v/v) %
1	Mucin	--	5 % (w/v)	0.5 %
2	Blood	--	100 % (v/v)	5 %
3	Afrin	Oxymetazoline	100 % (v/v)	10 %
4	Corticosteroids	Fluticasone	100 % (v/v)	5 %
5	Saline nasal spray	NaCl 0.9%	0.9 % (v/v)	10 %
6	Otrivin	Xylometazoline	100 % (v/v)	10 %

9.6 Clinical Specimen Stability

Clinical Specimen Stability was assessed by using contrived samples at the concentration of 3x LoD. The contrived samples were put through up to 3 freeze-thaw cycles before testing. Triplicate samples of each were tested using the MiRXES Fortitude SARS-CoV-2 & FluA/B Test by extracting 200 µL of each samples, eluting the resulting nucleic acid in 60 µL elution buffer, and 5 µL was used as input for the reactions. Extraction was performed using QIAamp Viral RNA Mini Kit. RT-qPCR was performed using Bio-Rad CFX96 Real-Time PCR Systems. Clinical specimens are stable for up to 3 freeze-thaw cycles in MiRXES Fortitude SARS-CoV-2 & FluA/B Test. Results are summarized in Table 15.

Table 15: Clinical Specimen Stability

	SARS-CoV-2			**Flu A**			**Flu B**		
	C_q value	Average	% shift	C_q Value	Average	% shift	C_q Value	Average	% shift
Baseline	32.20	32.20	N/A	33.13	33.10	N/A	28.67	29.35	N/A
	31.93			32.36			30.40		
	32.48			33.80			28.99		
1 F/T	31.94	32.21	0.01	32.89	32.57	-1.60	30.61	29.68	1.12
	32.42			32.81			30.16		
	32.26			32.00			28.28		
2 F/T	32.52	32.21	0.03	32.06	32.35	-2.25	29.97	29.87	1.77
	32.36			32.22			29.83		
	31.76			32.78			29.82		
3 F/T	32.55	32.00	-0.63	32.86	32.53	-1.70	28.71	28.62	-2.51
	31.77			32.41			28.57		
	31.68			32.33			28.57		

9.7 Clinical Evaluation

Clinical performance of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test was evaluated using 50 individual natural SARS-CoV-2 positive and 38 individual natural SARS-CoV-2 negative samples from nasopharyngeal swabs; 60 individual natural Flu A positive, 29 individual natural Flu B positive , and 26 individual natural Flu A and Flu B negative clinical specimens from a mixture of nasopharyngeal swabs and throat swabs.

In a blinded fashion, the specimens were extracted using QIAamp Viral RNA Mini kit. 200 µL of specimen was used for extraction. The elution volume was 60 µL. The extracted RNA was run on the MiRXES Fortitude SARS-CoV-2 & FluA/B Test using Bio-Rad CFX96 Real-Time PCR System. Results were compared with an EUA-approved SARS-CoV-2 detection kit and a FDA-approved FluA/B test kit.

Positive and Negative calls were made according to data interpretation from the MiRXES Fortitude SARS-CoV-2 & FluA/B Test IFU. Positive and Negative agreements were calculated based on the result generated from the comparators. The summary of result is shown in Table 16. The overall Percent Agreement was 100 %. The MiRXES Fortitude SARS-CoV-2 & FluA/B Test had been validated on samples collected in COPAN UTM® 3 mL, Puritan® UniTranz-RT Universal Transport Solution, and BD Universal Viral Transport Medium with extraction.

Table 16: Clinical performance

Virus	No. of samples	Comparator				% Agreement (95% CI)	
		Concordant Positive (N)	Discordant Positive (N)	Concordant Negative (N)	Discordant Negative (N)	PPA	NPA
SARS-CoV-2	88	50	0	38	0	100% (91.1% - 100%)	100% (88.6% - 100%)
Flu A	115	60	0	55	0	100 (92.5% - 100%)	100 (91.9% - 100%)
Flu B	115	29	0	86	0	100 (85.4% - 100%)	100 (94.7% - 100%)

10 Assay Limitations

- Strict compliance with the instructions for use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR, including testing procedures and interpretation of results prior to performing the assay.
- Good laboratory practice is essential for proper performance of this assay. Extreme care should be taken to preserve the purity of the components of the Kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of RT-PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- A false negative result may occur if inadequate numbers of the target organism (SARS-CoV-2, Influenza A, influenza B) are present in the specimen due to improper collection, transport or handling.
- As with any test, results of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test need to be interpreted in consideration of all clinical and laboratory findings.
- RNA viruses in particular show substantial genetic variability. Although continuous efforts were made to monitor potential mutation in the target regions that might result in mis-matches between the primers probes and the target sequences based on available viral sequences, new mutation might result in diminished assay performance and possible false negative results.

11 Quality Control

- To ensure consistent product quality, each lot of the MiRXES Fortitude SARS-CoV-2 & FluA/B Test is tested against predetermined specifications.
- Users are strongly discouraged to combine components from assay kits of different lot numbers.

12 Technical Assistance

For technical advice, please contact Technical Support:

fortitude@mirxes.com

13 Disclaimers

MiRXES Fortitude SARS-CoV-2 & FluA/B Test should only be used for the intended purpose and in accordance with the Instructions for Use.

MiRXES Pte Ltd is not liable for any damage or loss that may result from your use of the test.

14 Explanation of Symbols and Abbreviations

	Catalogue number		Manufacturer
	Caution		Batch Code
	Consult instructions for use		Temperature Limit
	*In Vitro* Diagnostic medical device		Use-by date
	Maximum 200 reactions		

MiRXES Pte Ltd
2 Tukang Innovation Grove
JTC MedTech Hub
#09-02
Singapore 618305