PlexPCR® Flu/RSV/SARS-CoV-2

Multiplex real-time RT-PCR assay for the detection of Influenza A, Influenza B, RSV and SARS-CoV-2

CE 0123 IVD
IVDR Certified

Product	Platform	Tests	Catalogue no.
PlexPCR® Flu/RSV/SARS-CoV-2	QuantStudio 5	192	REF 1703192

EC REP

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FOR PROFESSIONAL USE ONLY

Not for sale in the USA

Contents

1 Device description and test principles

The ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit is a single well multiplex RT-PCR for the detection and differentiation of Influenza A virus (Flu A, targeting the matrix protein gene), Influenza B virus (Flu B, targeting the matrix protein gene), Respiratory Syncytial Virus (RSV) type A & B (targeting the nucleocapsid gene), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2, targeting the ORF1ab and RdRP genes) and an endogenous cellularity control (EC) targeting the human Transferrin receptor protein 1 (TFRC) gene.

This test was validated using samples extracted using the KingFisher Flex System (Thermo Fisher Scientific) and real-time detection on the QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific).

2 Intended Use

The ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit is an *in vitro* diagnostic reverse transcriptase real-time PCR (RT-qPCR) test for the qualitative detection of Flu A, Flu B, RSV A, RSV B and SARS-CoV-2.

The ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit is intended to aid in the diagnosis of Flu A, Flu B, RSV A, RSV B and SARS-CoV-2 by a physician and should be used in conjunction with other clinical and laboratory information.

The ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit may be used with the following specimen type: nasopharyngeal swabs only.

The ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit is intended to be used in professional settings such as hospitals, reference, or state laboratories. It is not intended for self-testing, home use, near patient or point-of-care use.

The intended target population for the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit are symptomatic patients suspected of having respiratory viral infection by their healthcare provider based on clinical presentation and/or history.

3 Pathogen Information

Influenza viruses belong to the family Orthomyxoviridae and have a single stranded RNA genome. The influenza viruses are classified into types A, B, C, and D based on their core proteins, with only type A (Flu A) and B (Flu B) causing human disease of any concern. Flu A viruses are further divided into subtypes according to the specific combination of two proteins that occur on the surface of the virus (the hemagglutinin or "H" protein and the neuraminidase or "N" protein). Flu B can be divided into 2 groups (lineages), referred to as B/Yamagata and B/Victoria lineages. Influenza infections are characterized by a sudden onset of fever, cough (usually dry), headache, muscle and joint pain, severe malaise, sore throat, and a runny nose[1,2]. Most people recover from fever and other symptoms within a week without requiring medical attention, but influenza can cause severe illness or death especially in elderly, in infants and young children, and in immunocompromised hosts.

Respiratory syncytial virus (RSV) is a single-stranded RNA genome belonging to the Paramyxoviridae family Pneumovirus genus. Two subgroups exist, RSV A and RSV B[3]. RSV causes significant morbidity and mortality in infants worldwide, and accounts for as much as 70% of all childhood respiratory infections. In children up to 18 months old, RSV infection is associated with moderate to severe upper and lower respiratory tract infections, with symptoms including acute bronchiolitis, reactive airway disease, and excessive mucus production[4,5]. While RSV is mostly regarded as a childhood virus, adults are also susceptible to infection.

SARS-CoV-2 is a beta-coronavirus which emerged in China in late 2019. It has subsequently spread worldwide, resulting in a pandemic with significant morbidity and mortality. From December 2019 to June 2023, it is estimated that there have been more than 700 million confirmed cases of SARS-CoV-2 infection, resulting in more than 6.9 million deaths[6]. Most people infected with the virus will experience mild to moderate respiratory illness and recover without requiring special treatment. However, some will become seriously ill and require medical attention. Older people and those with underlying medical conditions like cardiovascular disease, diabetes, chronic respiratory disease, or cancer are more likely to develop serious illness[7].

4 Kit contents

Number of tests: 192 tests

Table 1. Kit contents for *Plex*PCR® Flu/RSV/SARS-CoV-2

Cap colour	Contents	Description	Quantity*
Green	***Plex*** Mastermix, 2x	Mastermix containing components necessary for qPCR including dNTPs, $MgCl_2$, DNA polymerase and buffer	2 x 1.2 mL
Yellow	Flu/RSV/SARS Mix, 20x^	Mix containing oligonucleotides (PCR primer pairs, ***Plex****Zyme*® enzymes and fluorescent probes) for amplification and detection of Flu A (single target), Flu B (single target), RSV (single target for RSV A, single target for RSV B), SARS-CoV-2 (dual target) and EC (single target).	1 x 230 µL
Black	RNase Inhibitor, 50x	Recombinant enzyme to inhibit RNase activity	1 x 135 µL
Neutral	RTase, 100x	Reverse transcriptase enzyme for generating complementary DNA (cDNA) from RNA template	1 x 90 µL

* Sufficient for 192 X 20 µl tests. Additional volume supplied for compatibility with liquid handling instrumentation

^ The Flu/RSV/SARS Mix, 20x contains fluorescent probes which are light sensitive. The mix is provided in an opaque, brown-coloured tube to protect contents from light.

5 Shipping and Storage

- The components of the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit are shipped on dry ice or ice gel packs. All components should be stored between -25°C to -15°C upon receipt. It is recommended that freeze/thaw cycles of the Flu/RSV/SARS mix are limited to 12.
- When stored under the recommended conditions and handled correctly, the activity of the kit is retained until the expiry date stated on the label. Do not use past expiry date.

6 Warnings and Precautions

6.1. General

- For *in vitro* diagnostic use only.
- Carefully read these Instructions for Use prior to use. Closely follow procedures as described to ensure reliability of test results. Any deviations from these procedures may affect test performance.
- Users should be adequately trained in the use of the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 assay.
- Any serious incident shall be reported to the manufacturer and competent authority of the Member State in which user and/or patient is established.

6.2. Laboratory

- It is recommended to perform sample preparation/extraction, mastermix preparation, sample addition and thermocycling in spatially separated spaces. At a minimum the PCR instrument should ideally be in a separate room to areas where tests are prepared.
- It is recommended to follow routine laboratory precautions. Wear appropriate personal protective equipment such as gloves, protective eye wear and laboratory coat when handling reagents.
- Pathogenic organisms may be present in clinical specimens. Treat all biological specimens as potentially infectious and follow your institution's safety procedures for handling chemicals and biological samples.
- Follow your institution's hazardous waste disposal procedures for proper disposal of specimens, reagents, and other potentially contaminated materials.

6.3. Specimen handling

- Specimens should be collected, transported, and stored using standard laboratory techniques or according to collection kit instructions. Refer to handling **Section 13.1** (Sample collection, transport, and storage)

6.4. Assay

- Basic precautions for preventing contamination of PCR tests include the use of sterile filter pipette tips, use of a new pipette tip for every pipetting action, and separation of workflow.
- PCR tests are prone to contamination from previous PCR products. Never open reaction vessels after the completion of PCR.
- The combined master mix containing Flu/RSV/SARS mix, 20x; RTase; RNase Inhibitor and Plex Mastermix component is stable at room temperature for period of 2 hours. Thus, this product should be used within 2 hours once thawed.

6.5. Safety precautions

- Safety Data Sheets (SDS) are available on request. Please contact tech@speedx.com.au for more information.

7 Associated Products and Consumables

*(The following products and consumables are not included with the **PlexPCR**® Flu/RSV/SARS-CoV-2 kit)*

External Positive Control

Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006). Refer to **Section 11.1** for instructions for use.

Sample Collection Devices

- 3 mL Universal Transport Medium with Regular FLOQ Swab - compatible with below swabs (Copan, Cat no 346C)
- MicroTest™ M4RT 3 mL Flocked Swab Kit (Thermo Scientific, Cat no. R12588)

General lab consumables

- Gloves and clean lab coats
- Vortex mixer
- Benchtop centrifuge for 0.5 mL and 1.5 mL tubes
- Micropipettors
- Multichannel pipettors
- Sterile aerosol-resistant pipette tips
- 0.5 mL tubes and 1.5 mL tubes (PCR-grade)
- Sterile 15 mL and 50 mL conical tubes
- Nuclease-free Water (not DEPC-Treated)
- Copan Universal Transport Medium (UTM) (Copan, Cat no 330C; refer to **Section 11.1**)

For KingFisher Flex™

- MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit (Thermo Fisher Cat no A48383)
- KingFisher 96 deep-well plate, v-bottom, polypropylene (Thermo Fisher Cat no 95040450)
- KingFisher 96 tip comb for deep-well magnets (Thermo Fisher Cat no 97002534)
- KingFisher 96 microplate (200 µL) (Thermo Fisher Cat no 97002540)
- 80% molecular grade ethanol
- 10 mL or 25 mL Reagent Reservoirs for 8 channel pipettes
- 50 mL Falcon Tubes

For QuantStudio 5™

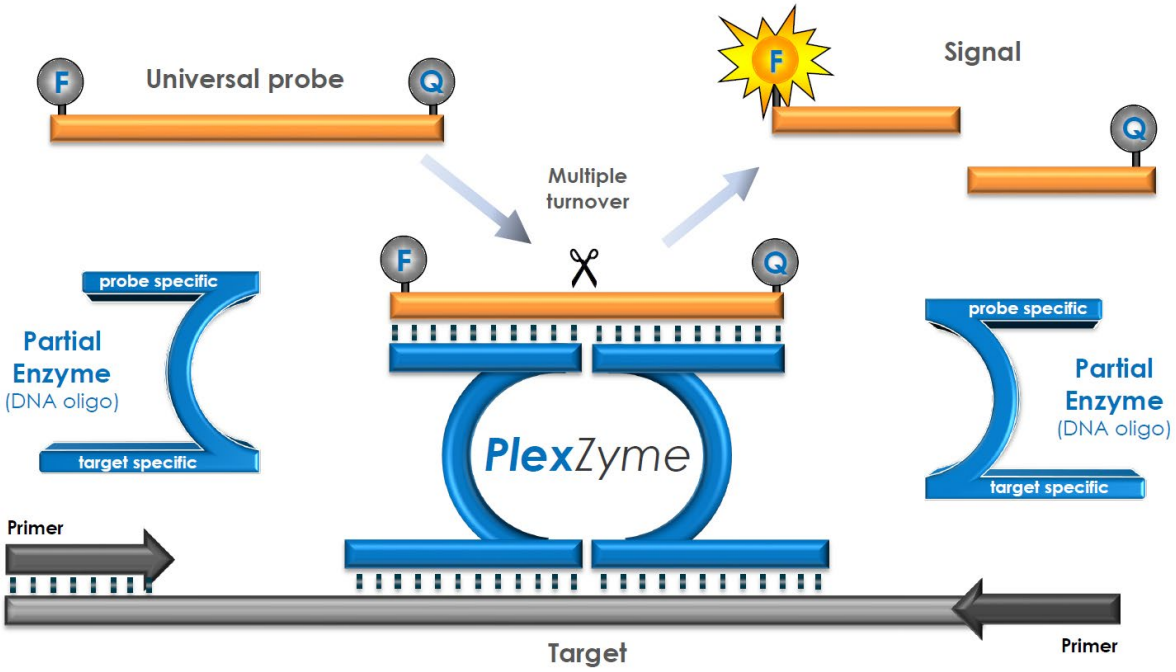
- MicroAmp™ Optical Adhesive Film (Thermo Fisher Cat no 4311971)
- MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode, 0.2 mL (Thermo Fisher Cat no 4483354)

8 Principle of the Technology

Real-time PCR (qPCR) can be used to amplify and detect specific target nucleic acids from pathogens. ***Plex****PCR*® is a qPCR technology utilising ***Plex****Zyme*® enzymes that detect and report the amplified product through the generation of a fluorescent signal (**Figure 1**).

Plex*Zyme*® enzymes are catalytic DNA complexes composed of two DNA oligos referred to as "Partial Enzymes". Each Partial Enzyme has a target-specific region, a catalytic core and a universal probe binding region. When the target product is present, the two Partial Enzymes bind adjacently to form the active ***Plex****Zyme*® which has catalytic activity to cleave a labelled probe. Cleavage separates the fluorophore and quencher dyes, producing a fluorescent signal that can be monitored in real time. ***Plex****Zyme*® enzymes have additional specificity compared to alternate detection technologies, since two Partial Enzymes are required to bind for detection. ***Plex****Zyme*® enzymes are also multiple turnover enzymes, and multiple probes can be cleaved during each PCR cycle, resulting in a strong and sensitive signal. ***Plex****Zyme*® assays are highly sensitive and specific and are ideally suited for the multiplexed detection of pathogens.

Figure 1. Schematic representation of *PlexZyme*® detection and universal signalling



9 Limitations

The ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay is suitable for the detection and differentiation of Influenza A virus (Flu A), Influenza B virus (Flu B), Respiratory Syncytial Virus (RSV) type A & B and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).

- The ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit should only be performed by personnel trained in the procedure and should be performed in accordance with the Instructions for Use.
- Reliable results are dependent on adequate specimen collection transport, storage, and processing. Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- The ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay is a qualitative assay and does NOT provide quantitative values or information about organism load.
- Results from the test must be correlated with the clinical history, epidemiological data, laboratory data and any other data available to the physician.
- Prevalence of viral targets will affect the positive and negative predictive values for the assay.
- Negative results do not exclude the possibility of infection due to improper specimen collection, technical error, presence of inhibitors, specimen mix up, or low numbers of organisms in the clinical specimen.
- False positive results may occur due to cross-contamination by target organisms, their nucleic acids or amplified product.
- Performance of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay has only been assessed using the validated specimen collection devices, sample extraction and real-time PCR detection systems. The use of a different methodology would require further validation by the user.
- The ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay may not identify different Flu B strains when the virus RNA is present in lower concentrations.
- Clinical samples with Cq value < 6 may not give a valid result. This will not be flagged by the QuantStudio 5 instrument software. The user must review all curves to ensure the baseline is within the flat-horizontal part of the curve before proceeding. See **Section 14.3** for more information on data interpretation. When a high load sample exceeds the detection limit, it is recommended to dilute and repeat the test.
- The instrument operating instructions provided in **Section 14.1 and Section 14.2** were created with the latest software version available at the time of preparation. Users should refer to the instructions of the most recent version provided by the instrument manufacturer. Please contact tech@speedx.com.au for any questions or further information.

10 Quality Control

The ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit includes an endogenous control (TFRC gene) assay to monitor sample adequacy, extraction, and PCR efficiency.

The Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006) is recommended as an external positive control. External positive controls are used for routine Quality Control testing to aid the user in detection of unexpected conditions that may lead to test errors. The detailed instructions are provided in **Section 11** for the Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006). A known negative specimen is recommended to be used as a negative control.

11 Positive Control Flu/RSV/SARS instructions

The Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006) is the recommended external positive control that has been validated for use as an external positive control with the ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit (Cat no 1703192).

The Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006) should be stored at 2-30°C until use. Once opened, the Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006) should not be reused.

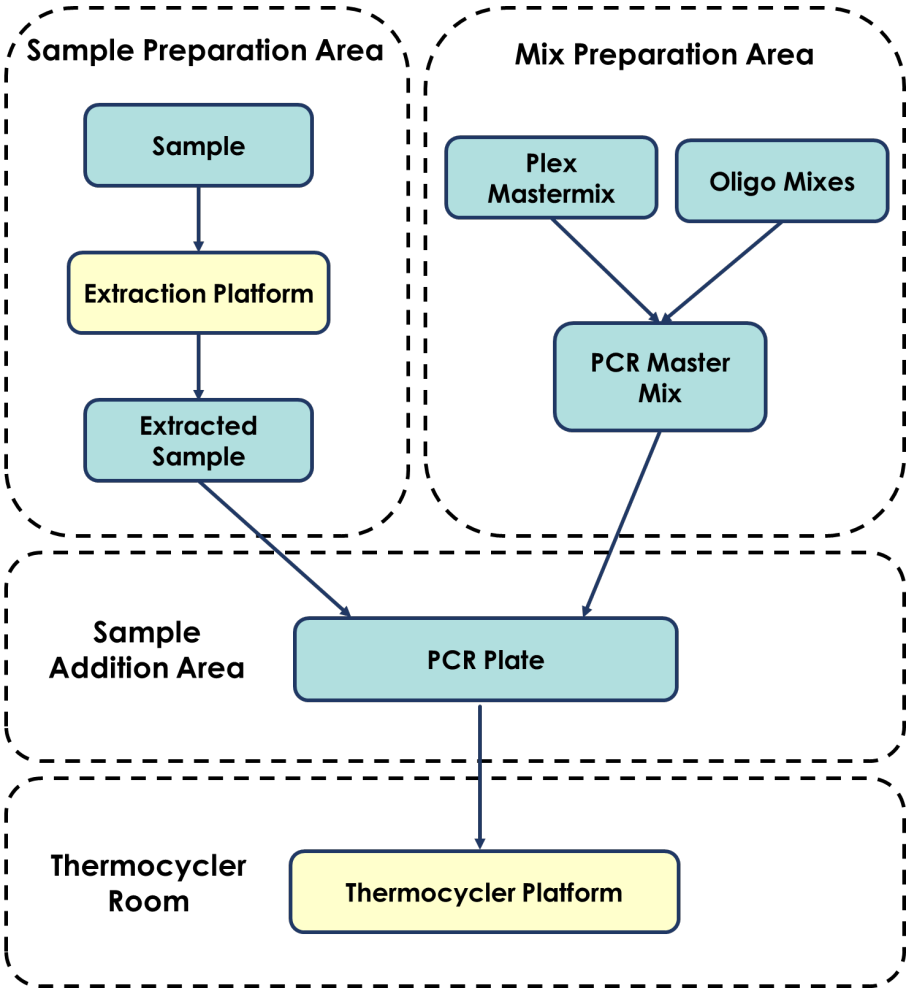
Please see the Positive Control Flu/RSV/SARS package insert for further information on storage and limitations.

11.1 Instructions for use

Prepare the SpeeDx Positive Control Flu/RSV/SARS in Copan Universal Transport Medium (UTM) (Copan, Cat no 330C) by submerging the swab tip in 3mL Copan UTM, then snapping the swab at the breakpoint before fastening the screw cap and incubating at room temperature for one minute. Vortex the vial with the tip submerged for 30 seconds. Once prepared, the PC eluted in UTM is stable for up to six days when stored at 2-8ºC.

Extract the positive control and prepare qPCR reactions as described in **Section 13** using positive control material as sample.

12 Procedure overview



13 Detailed procedure

Note: Provided reagents are named in italics and colour of the tube cap follows in brackets.

13.1 Sample collection, transport, and storage

Nasopharyngeal swabs should be collected and transported according to collection kit instructions.

13.1.1 Validated sample collection devices

Inadequate or inappropriate specimen collection, storage and transport are likely to yield false test results. Proper training in specimen collection is highly recommended to ensure specimen quality and stability.

Sample collection devices with the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 kit are included below with short guidance regarding the device manufacturer's instructions for collection, handling, and transport. These instructions are not intended to replace or supersede any instructions provided by the manufacturer. Always refer to specimen collection device manufacturer instructions for proper collection methods.

Prior to any collection method, trained staff must ensure proper understanding of the device and methodology. At minimum, review the test description for the following: indication of specimen type, sufficient volume, procedure(s), necessary collection materials, patient preparation, and proper handling and storage instructions.

13.1.1.1 Copan FLOQSwabs® in 3mL UTM™ media (Copan, Cat no 346C)

- Open the pouch from the side indicated by the arrow and remove the swab taking care not to touch anything with the swab tip.
- Collect the sample. During sampling, the swab tip must only come in contact with the area from which the specimen is to be taken in order to reduce contamination risks. NOTE: Do not bend the swab before the collection of the specimen. Do not use excessive force, pressure or bending when collecting swab samples from patients as this may result in accidental breakage of the swab shaft.
- Check that the test tube and swab sizes are compatible.
- After collecting the specimen, insert the swab into the test tube until the breakpoint is level with the test tube opening.
- Bend the swab shaft at a 180-degree angle to break it off at the breaking point. If needed, gently rotate the swab shaft to complete the breakage and take away the upper part of the swab shaft.
- Discard the broken handle part of the swab shaft into an approved medical waste disposal container.
- Screw the cap back onto the test tube and hermetically seal it.
- After collection, the specimen should be tested immediately. If there is any delay in processing of samples, they can be stored at ambient room temperature (15°C to 20°C) for up to 2 hours, at refrigerated temperatures (2°C to 8°C) for up to 7 days, frozen (-25°C to -15°C) for up to 5 days and stable up to 3 F/T cycles. Samples can also be stored up to 12 months at -80°C and are stable for 1 freeze thaw cycle.

13.1.1.2 MicroTest™ M4RT 3 mL Flocked Swab Kit (Thermo Scientific, Cat no. R12588)

- Aseptically remove cap from vial.
- Insert swab into medium.
- Break swab shaft evenly at the scored line. Use sterile pair of scissors if additional trimming is needed.
- Replace cap to vial and close tightly.
- Label with appropriate patient information.
- Send to the laboratory for processing with minimal delay.

After collection, the specimen should be tested immediately. If there is any delay in processing of samples, they can be stored at ambient room temperature (15°C to 20°C) for up to 2 hours, at refrigerated temperatures (2°C to 8°C) for up to 7 days, frozen (-25°C to -15°C) for up to 5 days and stable up to 3 F/T cycles. Samples can also be stored up to 12 months at -80°C and are stable for 1 freeze thaw cycle.

13.2 Sample processing

All samples are to be extracted using the KingFisher Flex Magnetic Particle Processor according to the manufacturer's instructions. The extraction kit and protocol details are outlined in **Table 2**.

A positive and negative control must be extracted and included in every run, see **Section 11** for instructions to use the SpeeDx Positive Control Flu/RSV/SARS (SpeeDx, Cat no 95006).

Table 2. Extraction protocols

Instrument	Extraction kit	Sample volume	Protocol	Elution volume
KingFisher Flex[a]	MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit	200 µL	MVP_2Wash_200_Flex	50 µL

[a] Samples should be added to the Mastermix within 30 minutes following extraction.

13.2.1 Reagent volumes for KingFisher Flex

The reagent volumes per sample for the KingFisher Flex are listed in **Table 3.**

Table 3. KingFisher reagent volumes

Reagent	Volume per sample	Plate
MagMax Binding Solution	265 µL	KingFisher 96 deep-well plate (sample plate)
MagMax Total Nucleic Acid Binding Beads	10 µL	KingFisher 96 deep-well plate (sample plate)
MagMax Proteinase K	5 µL	KingFisher 96 deep-well plate (sample plate)
MagMax Wash Solution	500 µL	KingFisher 96 deep-well plate
Wash 1 (80% Ethanol)*	500 µL	KingFisher 96 deep-well plate
MagMax Elution Buffer	50 µL	KingFisher 96 microplate 200 µL

* Not supplied within MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit

Instructions for programming the KingFisher Flex system are outlined in **Section 14.1.**

13.3 Endogenous Control (EC)

The Flu/RSV/SARS mix (**YELLOW**) includes an endogenous control (TFRC gene) assay to monitor sample adequacy, extraction, and PCR efficiency.

13.4 Preparation of real-time PCR

Note: Before use of reagents, thaw completely, and mix thoroughly by briefly vortexing for 1-3 seconds followed by centrifuging for 1-3 seconds. Refer to **Table 1** for a description of kit contents.

13.4.1 Master Mix preparation

For a 20 µL reaction volume, 11.6 µL of Master Mix and 8.4 µL of extract is required.

Prepare Master Mix as outlined in **Table 4**. Once prepared, the combined Master Mix should be vortexed and centrifuged for 1-3 seconds before adding to the PCR plate. Pipette 11.6 µL of Master Mix into each well of the PCR plate and then add 8.4 µL of extracted sample to the reaction.

- One positive control and one negative control should be run on each plate.
- Seal, then centrifuge the plate and transfer to the thermocycler.

Table 4. Master Mix

Reagent	Concentration	Volume per 20 µL reaction (µL)
Plex Mastermix (**GREEN**)	2x	10.0
Flu/RSV/SARS mix (**YELLOW**)	20x	1.0
RTase (**NEUTRAL**)	100x	0.2
RNase inhibitor (**BLACK**)	50x	0.4
Total volume (µL)		11.6
Add 8.4 µL sample for a final volume of 20 µL		

Instructions for programming the QuantStudio™ 5 Real-Time Detection System are outlined in **Section 14.2.**

14 Programming and analysis

14.1 Programming the KingFisher Flex System

The following information is based on the KingFisher™ BindIt™ Software v4.1. These instructions are not intended to replace or supersede any instructions provided by the manufacturer. Always refer to manufacturer's instruction for proper and up-to-date instructions.

Execute the Protocol (**Figure 2**)

Make sure the instrument is turned off then connect to the PC with a USB cable.
Turn on the instrument
Double click the BindIt icon to launch the software. Click **Connect**. The software finds the instrument automatically.
Application Menu appears on the display

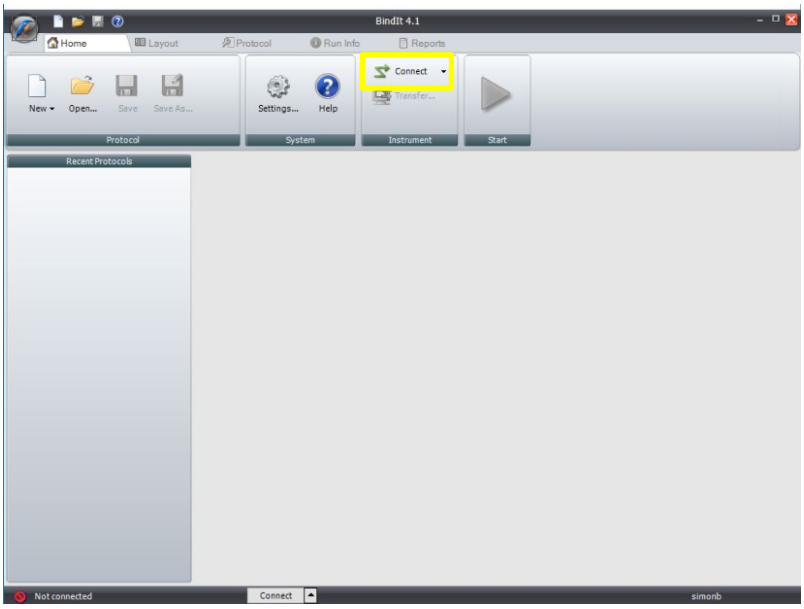


Figure 2. BindIt software display.

Click **Open** to select the **MVP_2Wash_200_Flex** Protocol (**Figure 3**).

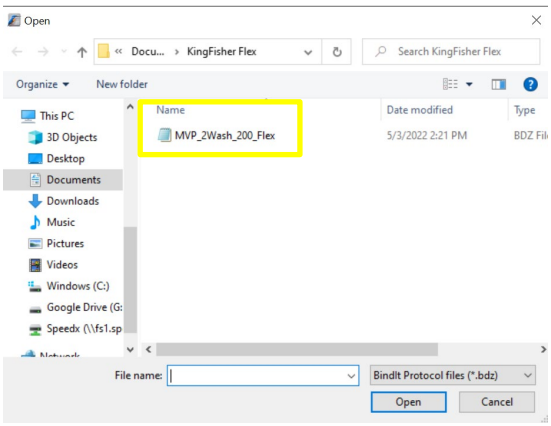


Figure 3. Selecting the MVP_2Wash_200_Flex protocol

Add Sample IDs **(Figure 4**)

Select the **Run Info** tab
Go to the **Samples** tab.

Click the arrow next to the **Sample** tab to add samples from a text file (.txt).
Set the fill direction.
Enter the number of replicates and their direction (if applicable).
Select the starting point in the layout.
Click **Add**.

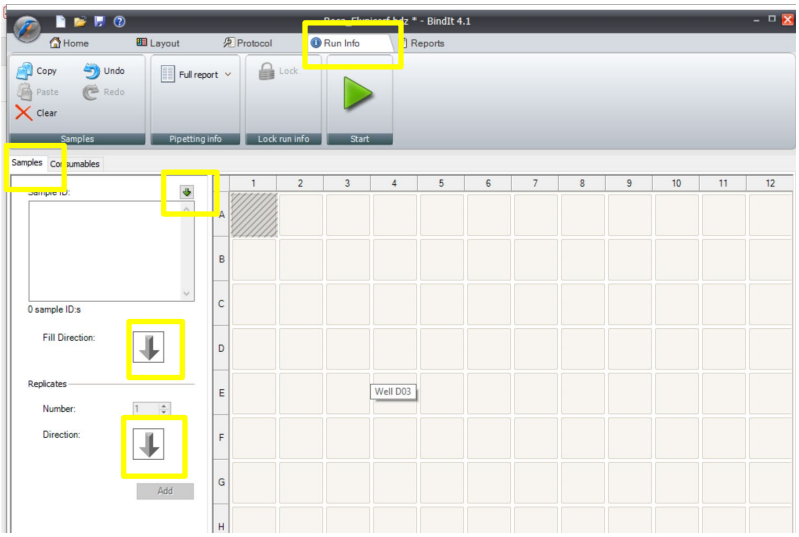


Figure 4. Adding sample ID information to the 'Samples' tab

Add Lot Information **(Figure 5)**
Go to the **Consumables** tab.
Tick the desired reagents.
Click **Add**.
Enter the lot number.
Click **OK**

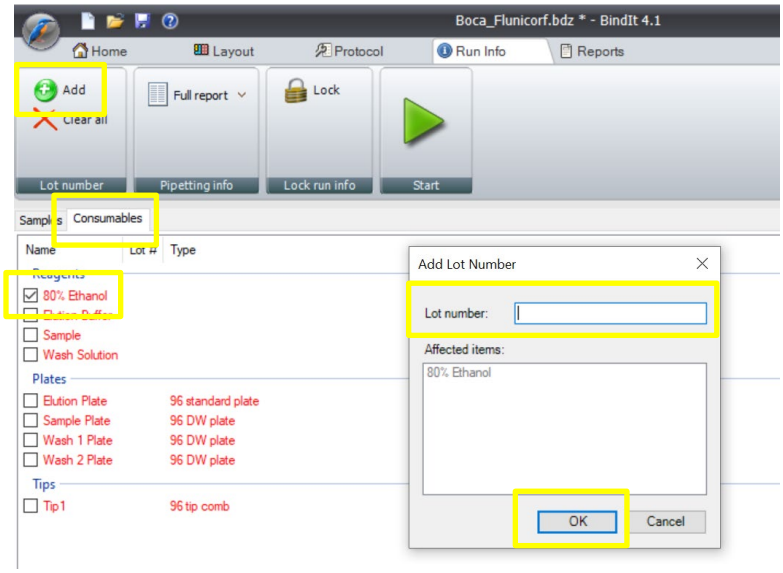


Figure 5. Adding Lot information to the 'Consumables' tab

Click **Start**  to launch the protocol

14.2 Programming the QuantStudio™ 5 Real-Time Detection System

The following information is based on QuantStudio™ Design & Analysis Software v1.5.2. These instructions are not intended to replace or supersede any instructions provided by the manufacturer. Always refer to manufacturer's instruction for proper and up-to-date instructions.

The ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit contains dyes for the Applied Biosystems® QuantStudio™ 5. Default dye calibrations are used for all channels. Custom calibration is not required.

Select **Create New Experiment**

In **Setup** > open **Properties** and select the following

Name the experiment

Instrument Type > QuantStudio™ 5 System

Block Type > 96-Well 0.2-mL Block

Experiment Type > Standard Curve

Chemistry > Other

Run Mode > Standard

In **Setup** > open **Method**

Set **Reaction Volume** > 20 µL

Create the following program outlined in **Table 5** (shown in more detail in Graphical View (**Figure 6** and **Figure 7**).

Ensure that 'AutoDelta' is enabled and fluorescence data acquisition is selected during the specified steps in **Table 5.**

Table 5. Thermocycling Program

Program name	Cycles	Target °C	Hold	Ramp≠
Reverse Transcriptase	1	48°C	10 min	1.6°C/s
Polymerase activation	1	95°C	2 min	1.6°C/s
Touch down cycling:	10	95°C	5 s	1.6°C/s
Step down -0.5°C/cycleδ		61°C – 56.5°Cδ	30 s	1.6°C/s
Quantification cycling+:	40	95°C	5 s	1.6°C/s
Acquisition/Detection		52°C+	50 s	1.6°C/s

≠ Default ramp rate

δ Enable AutoDelta: -0.5°C/cycle

+ Collect data on hold

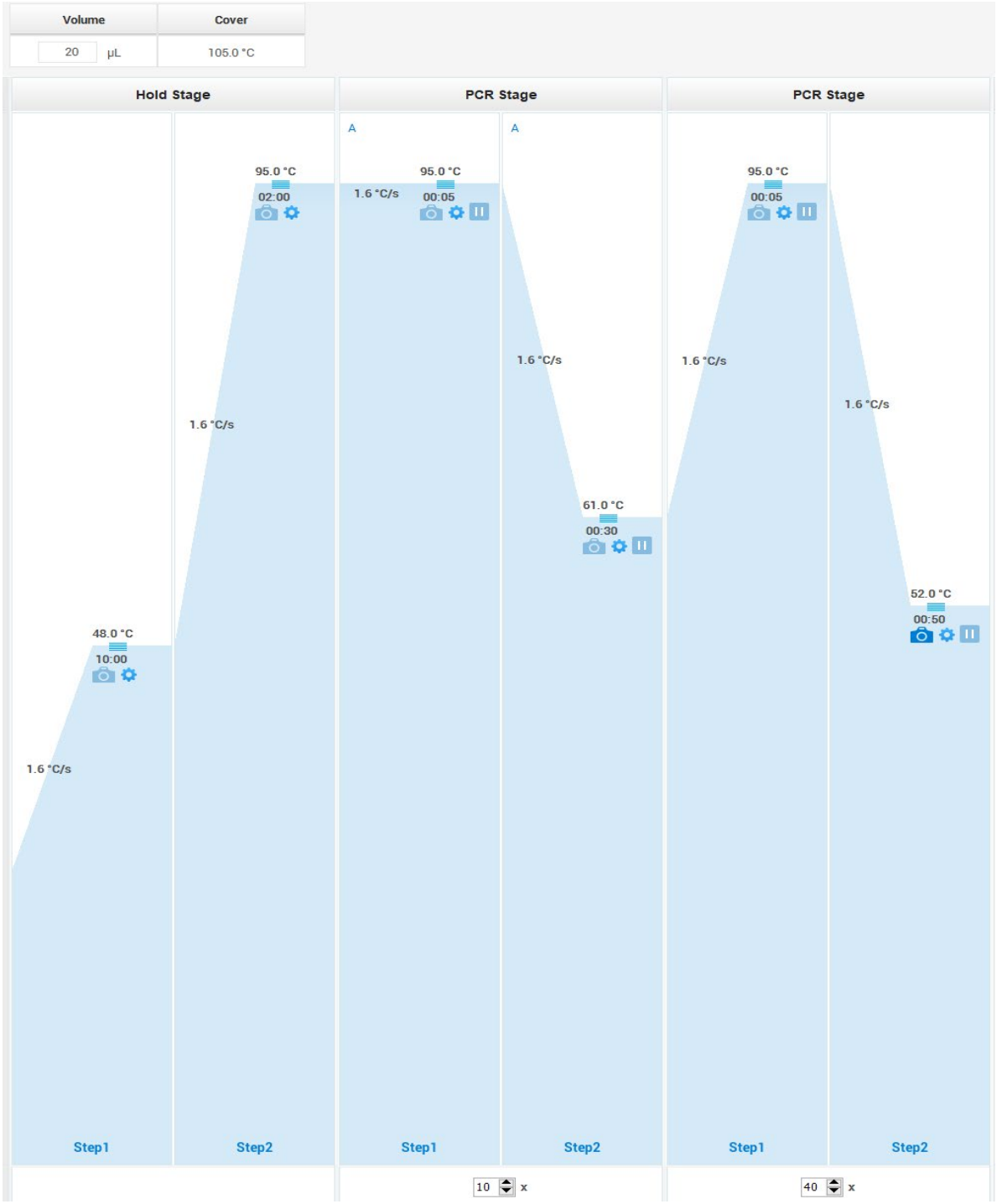


Figure 6. Run method – Graphical view

To enable 'AutoDelta' select the icon to open the Advanced Settings dialogue window (**Figure 7**).

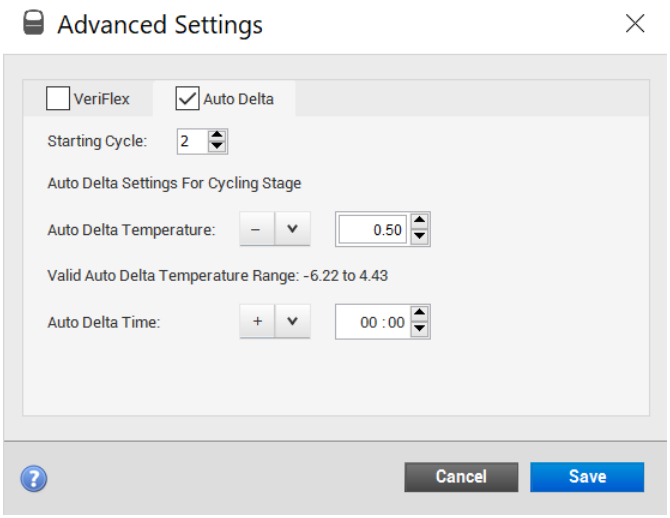


Figure 7. Run method – Graphical view – Enable AutoDelta

In **Setup** > open **Plate**

In the **Advanced Setup** tab **>**

Targets > Click Add to add new targets and define as shown in **Table 6** below (define colours as required)

Table 6. Define Targets

Target name	Reporter	Quencher
FluA	ROX	None
FluB	VIC	None
RSV	Cy5	None
SARS-CoV-2	FAM	None
EC (TFRC)	TAMRA	None

Samples >Click Add to add new samples and define the sample name

In the **Quick Setup** tab **>**

Plate attributes > Select Passive reference > None

Well attributes > Select wells and assign targets and samples to the selected wells

In **Setup** > open **Run**

Select **Start Run**

14.3 Analysing results with the QuantStudio™ Design & Analysis software

Data interpretation may be performed using the QuantStudio™ Design & Analysis software by applying the validated parameters provided below. The QuantStudio™ Design & Analysis software requires the user to set reporter dyes for each target; they can be set as listed in **Table 7**.

Table 7. Reporter Dyes for *PlexPCR*® Flu/RSV/SARS-CoV-2 targets				
Cy5	**FAM**	**VIC**	**ROX**	**TAMRA**
RSV	SARS-CoV-2	Influenza B	Influenza A	Endogenous Control

Analysis parameters are recommended based on QuantStudio™ Design and Analysis Software USER GUIDE (Publication Number MAN0010408, Rev. B.0).

14.3.1 Setting Analysis Parameters

Set baseline for each target

In the Results tab, view the baseline values. Click the Graph options icon and change the Graph Type to Linear (**Figure 8**).

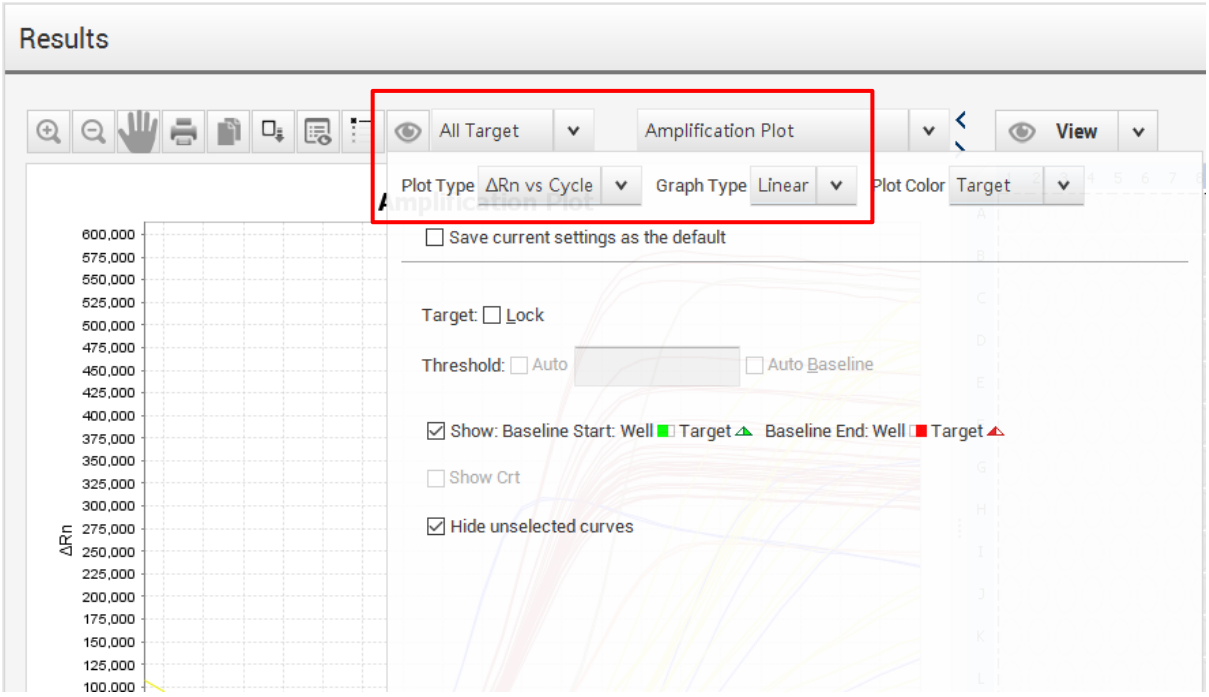
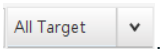
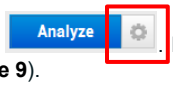


Figure 8. Setting the Amplification Plot Graph Type to Linear

View the results for each Target individually by selecting one channel at a time in the Target drop down menu 

.

The baseline cycle parameters should be set within the horizontal part of the baseline and should be set for each target independently. Manually setting the baseline start cycle to 4 and end cycle to 10 is recommended as a starting point for all targets and can be adjusted for each sample and target as needed so that they fall within the flattest part of the baseline range.

To change the baseline settings, click the settings “cog” icon to the right of the blue analysis button 

. Deselect the “Default Settings” option and then the “Automatic Threshold” and “Automatic Baseline” options (**Figure 9**).

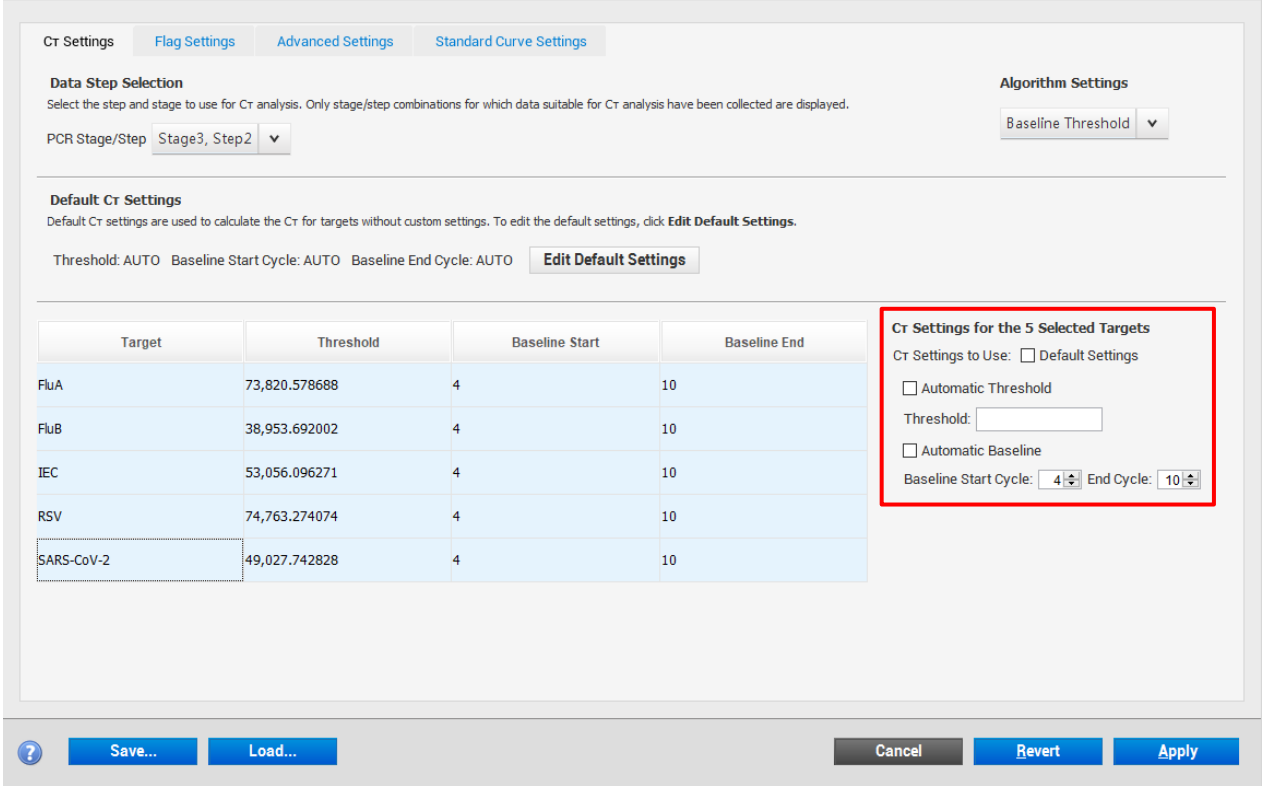


Figure 9. Changing the baseline and threshold options in the Ct Settings menu

NOTE: Before adjusting the baseline and threshold options, ensure that you have selected the target you wish to adjust, otherwise the software will automatically select the first target listed.

NOTE: For samples with a high load of target present and early Cqs (within the first 6 cycles), additional instructions are provided to assist with setting the baseline cycle parameters in **Section 14.3.2.**

Set threshold for each target

After the baseline cycle parameters are set, the threshold for each target can be set independently. Click and drag the fluorescence threshold to the maximum endpoint fluorescence value for that target channel (**Figure 10**).

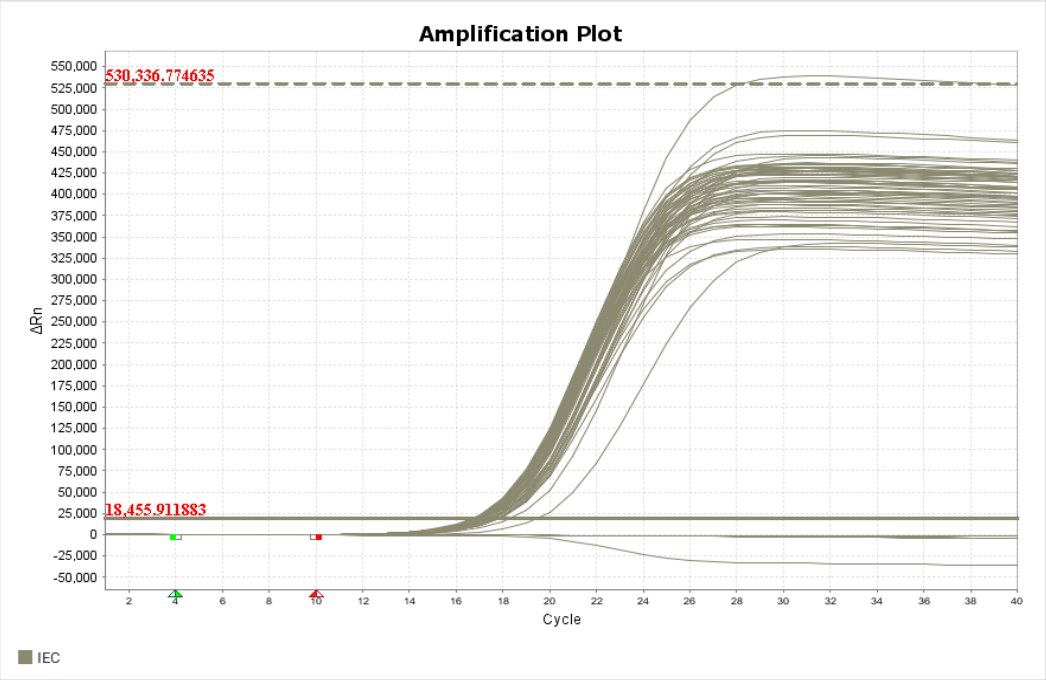
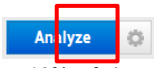


Figure 10. Click and drag the threshold to the maximum endpoint fluorescence value.

To set the threshold value, click the settings "cog" icon to the right of the blue analysis button 

. The software will report this endpoint fluorescence value for that channel (**Figure 9**). Adjust the threshold to be 10% of the maximum endpoint fluorescence value it reported (move decimal one place to the left and click the Apply button) 

(**Figure 11**).

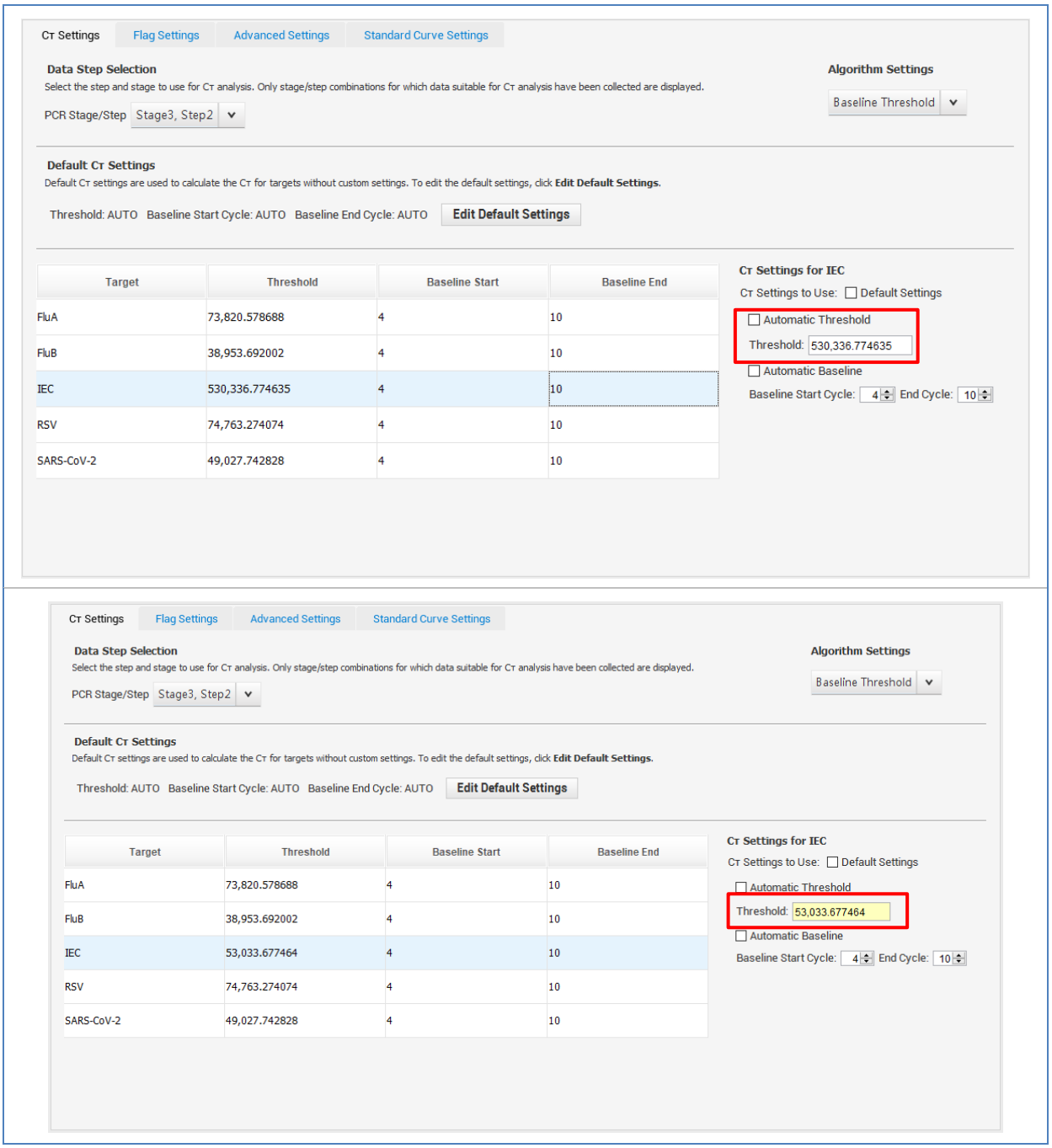


Figure 11. Setting the fluorescence threshold. Top - after manually adjusting the threshold to the maximum endpoint fluorescence, the value is reported in the threshold box. Bottom - User can adjust the value to be 10% of the reported maximum endpoint fluorescence.

The fluorescence threshold for that channel is now set at 10% maximum endpoint fluorescence (**Figure 12**).

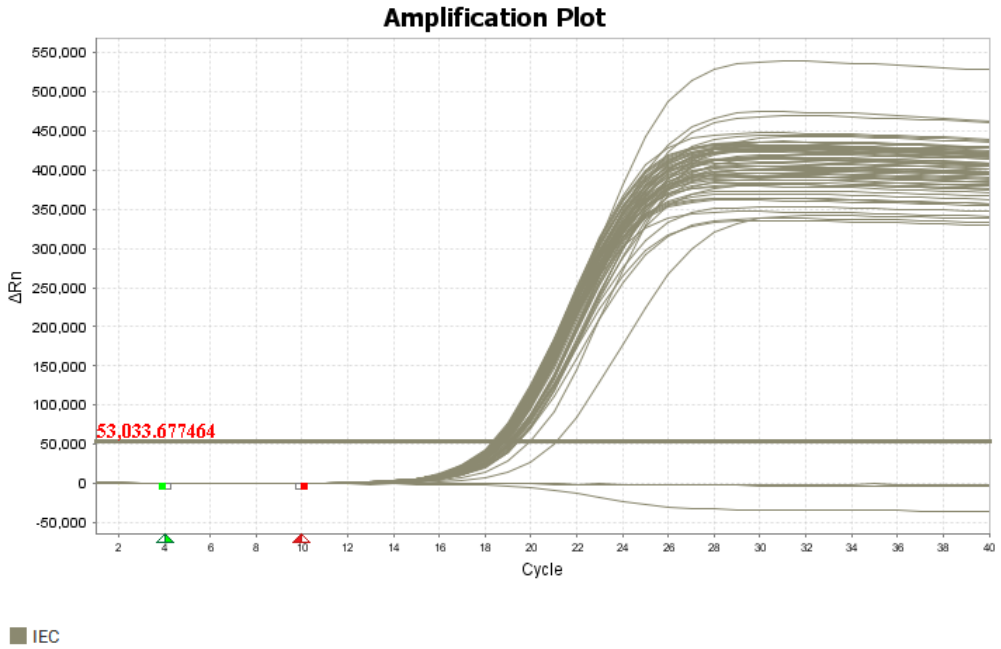


Figure 12. Fluorescence threshold is set to 10% of maximum endpoint fluorescence.

Repeat the process for each target. Once all parameters are set click the blue analysis button 

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14.3.2 Setting Analysis Parameters for high load samples

For samples with a high load of target present and early Cqs (within the first 6 cycles), there is a risk that the recommended baseline setting (4-10) results in an incorrect call. For these samples a narrower baseline setting range of 4-6 is recommended and can be further adjusted if needed as described in **Figure 13**.

In this example, the starting point of the curve in the FAM CHANNEL did not fall on the flattest part of the baseline range (after applying the recommended baseline setting: 4-10).

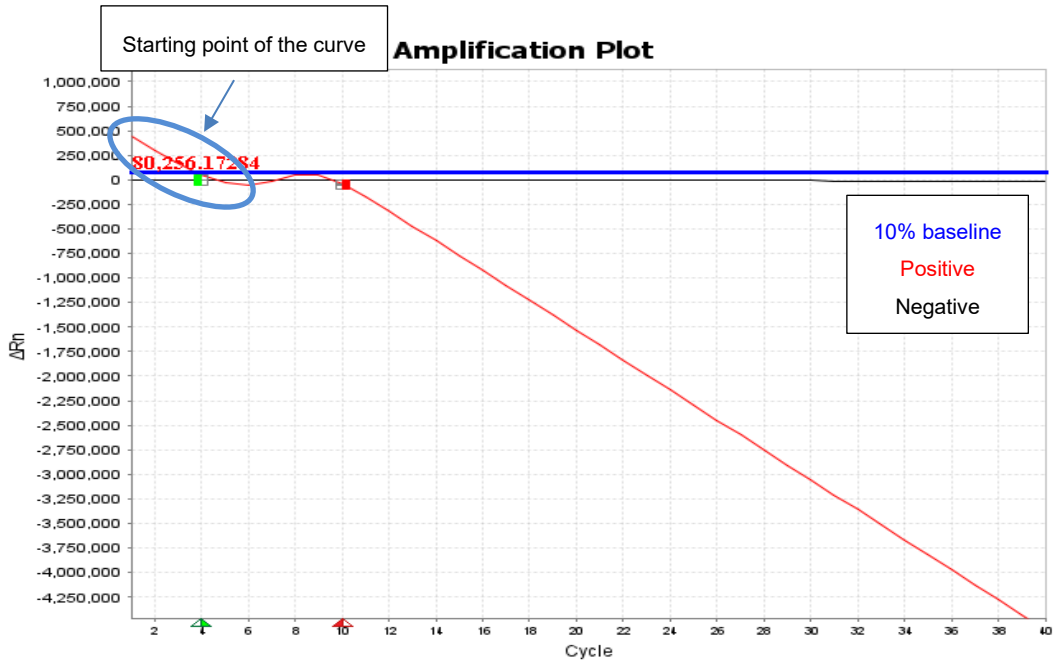

Figure 13. Example PCR curve of a high load sample

The FAM CHANNEL (SARS-CoV-2) was flagged as negative (no Ct) as the curve did not pass the 10% threshold.

It is recommended to adjust the baseline setting so that the starting point of the curve falls within the flattest part of the baseline range. The baseline start cycle should be ≤4 and the end cycle should be around the starting point of the exponential phase of the curve as show in **Figure 14 - Figure 16.**

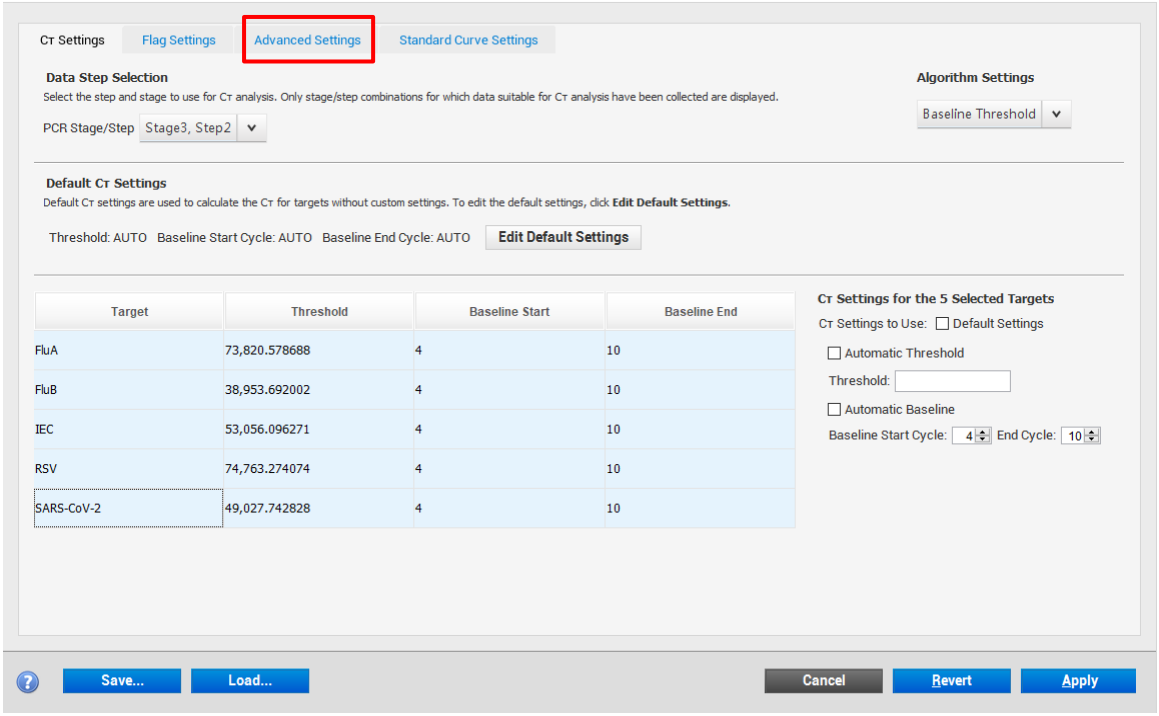

Figure 14. Changing the baseline for specific samples in Advance Settings

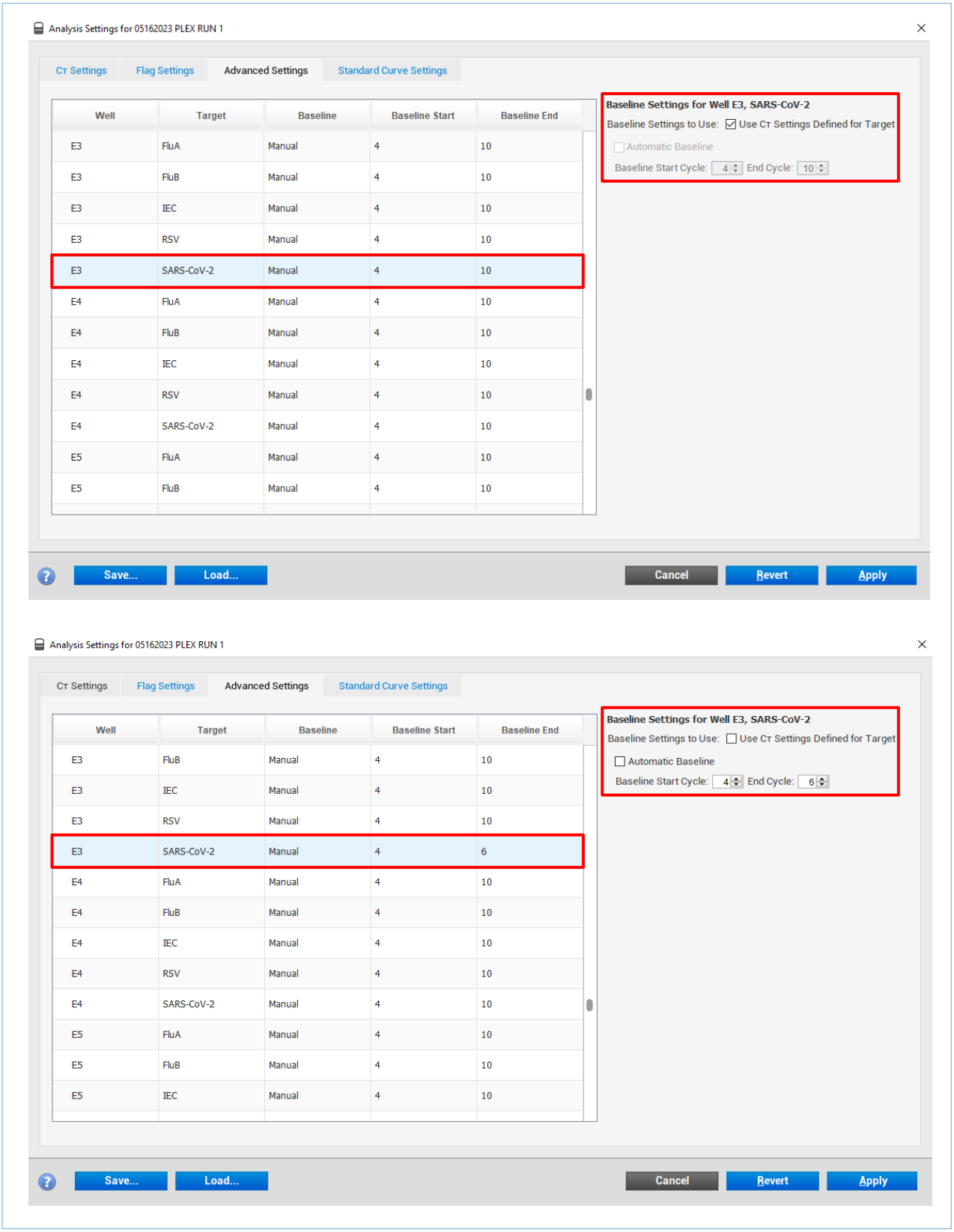

Figure 15. Setting the baseline for high load sample. Top - before adjusting the baseline start and end cycle (4-10). Bottom after adjusting the baseline start and end cycle (4-6).

#	Well	Sample Name	Target Name	Task	Dyes	Ct
51	E3	Sample 1	FluA	UNKNOWN	ROX-None	Undetermined
51	E3	Sample 1	FluB	UNKNOWN	VIC-None	Undetermined
51	E3	Sample 1	IEC	UNKNOWN	TAMRA-None	14.700
51	E3	Sample 1	RSV	UNKNOWN	CY5-None	Undetermined
51	E3	Sample 1	SARS-CoV-2	UNKNOWN	FAM-None	Undetermined

#	Well	Sample Na...	Target Na...	Task	Dyes	Ct
51	E3	Sample 1	FluA	UNKNOWN	ROX-None	Undetermined
51	E3	Sample 1	FluB	UNKNOWN	VIC-None	Undetermined
51	E3	Sample 1	IEC	UNKNOWN	TAMRA-None	14.700
51	E3	Sample 1	RSV	UNKNOWN	CY5-None	Undetermined
51	E3	Sample 1	SARS-Co...	UNKNOWN	FAM-None	5.779

Figure 16. The FAM CHANNEL (SARS-CoV-2) result changed from "undetermined" (SARS-CoV-2 negative) to a Ct of 5.779 (SARS-CoV-2 positive) as the curve passed the 10% threshold following the baseline setting adjustment.

14.3.3 Exporting Results

To export results as a Microsoft Excel file (.xlsx) file or tab delimited text file (.txt), select **Export** tab and ensure the Results option is selected. Choose the export location and click the Export button (**Figure 17**).

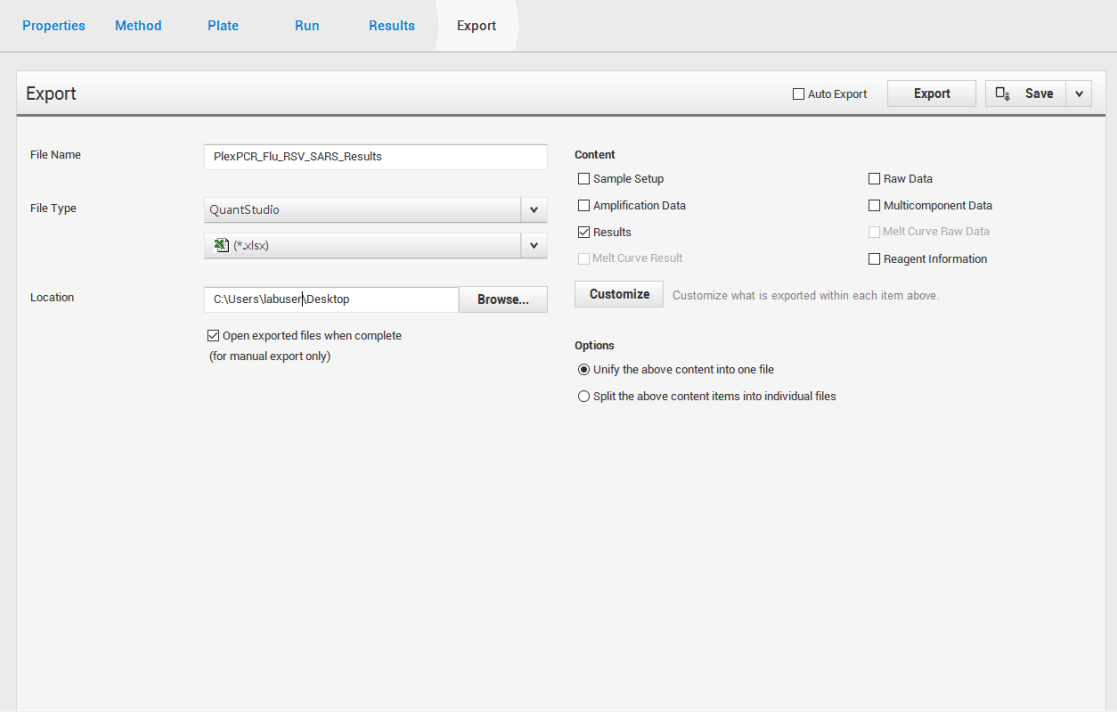

Figure 17. Exporting results from the QuantStudio™ Design & Analysis software.

15 Interpretation of results

For interpretation of results, each sample must be analysed individually. See **Table 8** for how to interpret signals from different channels. Any Cq registered within the cut-off, with visual confirmation of the amplification curve, is a positive result.

Negative Control (NC)

A negative control is valid if the TAMRA channel registers a Cq within the cut-off (**Table 8**) and all other channels do not produce a signal. If the negative control is not valid the result is INVALID, and extraction and PCR should be repeated.

Positive Control (PC)

A positive control is valid if there are positive signals in the Cy5, FAM, VIC and ROX channels and the Cq values fall within the cut-offs (**Table 8**). A valid positive control also requires no signal in the TAMRA channel within the cut off (**Table 8**). If the positive control is not valid the result is INVALID, and extraction and PCR should be repeated.

Endogenous Control

The endogenous control monitors extraction and sample adequacy. The endogenous control is valid if the TAMRA channel registers a Cq within the Cut-off (**Table 8**).

For samples where target assays are negative and the endogenous control assay is also negative, the result is INVALID, and the extraction and PCR should be REPEATED and check for contamination.

For samples where target assays are positive but the endogenous control assay is negative, the result is also INVALID and the samples should be RETESTED.

Table 8. Interpretation of Results					
	Target				
Interpretation	**RSV (Cy5)**	**SARS-CoV-2 (FAM)**	**Influenza B (VIC)**	**Influenza A (ROX)**	**Endogenous Control (TAMRA)**
RSV detected	Cq ≤40	-	-	-	Cq ≤ 30
SARS-CoV-2 detected	-	Cq ≤37	-	-	Cq ≤ 30
FluB detected	-	-	Cq ≤40	-	Cq ≤ 30
FluA detected	-	-	-	Cq ≤40	Cq ≤ 30
SARS-CoV-2, Flu A, Flu B, RSVA & RSVB not detected	-	-	-	-	Cq ≤ 30
EC invalid. Re-extract and re-test sample	-	-	-	-	- Or Cq > 30
	Interpretation of controls				
PC valid	Cq ≤40	Cq ≤37	Cq ≤40	Cq ≤40	- Or Cq > 30
NC valid	-	-	-	-	Cq ≤ 30

It is recommended the user starts with a manual baseline setting of 4-10 cycles, however clinical samples may appear invalid if they are of a high viral load, this may not be flagged by the on-board QuantStudio™ 5 software. The user must review all curves to ensure the baseline is within the flat-horizontal part of the curve before proceeding.

16 Performance Characteristics

16.1 Clinical performance

A retrospective clinical study was conducted at Boca Biolistics Reference Laboratory (BBL), Pompano Beach, Florida, United States, on characterised biobank and prospectively collected specimens from 2020-2022. A total of 688 nasopharyngeal specimens collected in UTM or Viral Transport Media (VTM) from symptomatic individuals were initially included in the study. 17 samples were excluded from the final results due to either an invalid EC result with ***PlexPCR***® Flu/RSV/SARS-CoV-2 or the comparator assay (13 samples), insufficient volume (3 samples) or not meeting the inclusion criteria after commencement of the study (1 sample). Samples were extracted using the KingFisher Flex Magnetic Particle Processor (ThermoFisher) using MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit. 200µl of sample was extracted and the final elution volume was 50µl. Samples were tested with the ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit in 20µl on the Applied Biosystems QuantStudio 5 (QS5).

The clinical performance of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit was compared to the PKamp™ Respiratory SARS-CoV-2 RT-PCR Panel 1 (PerkinElmer) for the detection of influenza A, influenza B, RSV and/or SARS-CoV-2. The sensitivity and specificity for the ***PlexPCR***® Flu/RSV/SARS-CoV-2 kit are shown in **Table 9** and **Table 10.** Discrepant analysis was performed using the BioFire® Respiratory Panel 2.1 (bioMerieux), results of the discrepant analysis are provided as a footnote to the performance tables below.

Table 9. Clinical evaluation of the *PlexPCR*® Flu/RSV/SARS-CoV-2 – Flu A & Flu B

		PKamp™ Respiratory SARS-CoV-2 RT-PCR Panel 1			
		Flu A		Flu B	
		Positive	Negative	Positive	Negative
***PlexPCR*® Flu/RSV/SARS-CoV-2**	**Positive**	84	6[1]	105	4[3]
	Negative	0	581	6[2]	556
Total		84	587	110	560
Sensitivity		100.0% (95% CI 95.7 - 100.0%)		94.6% (95% CI 88.6 – 98.0%)	
Specificity		99.0% (95% CI 97.8 – 99.6%)		99.3% (95% CI 98.2 – 99.9%)	

[1]Influenza A was detected in 5/6 FP specimens during discrepancy investigation using the BioFire Respiratory Panel 2.1. Influenza A was not detected by BioFire Respiratory Panel 2.1 in 1/6 FP specimens.
[2]5/6 FN specimens were negative for Influenza B when tested during the discrepancy investigation with BioFire.
[3]3/4 FP specimens were negative for Influenza B when tested during the discrepancy investigation with BioFire.

Table 10. Clinical evaluation of the *PlexPCR*® Flu/RSV/SARS-CoV-2 – RSV & SARS-CoV-2

		PKamp™ Respiratory SARS-CoV-2 RT-PCR Panel 1			
		RSV		SARS-CoV-2	
		Positive	Negative	Positive	Negative
***PlexPCR*® Flu/RSV/SARS-CoV-2**	**Positive**	106	6[2]	100	24[4]
	Negative	3[1]	556	1[3]	546
Total		109	562	101	570
Sensitivity		97.2% (95% CI 92.2 – 99.4%)		99.0% (95% CI 94.6 – 100.0%)	
Specificity		98.9% (95% CI 97.7 – 99.6%)		95.8% (95% CI 93.8 – 97.3%)	

[1]RSV was detected in all 3 FN specimens during discrepancy investigation by BioFire Respiratory Panel 2.1
[2]RSV was detected in 4/6 FP specimens during discrepancy investigation using the BioFire Respiratory Panel 2.1. 1/6 FP specimens was unable to be further investigated due to insufficient sample. RSV was not detected by BioFire Respiratory Panel 2.1 in 1/6 FP specimens.
[3]SARS-CoV-2 was detected in the single FN specimen during discrepancy investigation using the BioFire Respiratory Panel 2.1.
[4]SARS-CoV-2 was detected in 18/24 FP specimens during discrepancy investigation using the BioFire Respiratory Panel 2.1. 1/24 FP specimens was unable to be further investigated due to insufficient sample. SARS-CoV-2 was not detected by BioFire Respiratory Panel 2.1 in 5/24 FP specimens.

16.2 Analytical performance

16.2.1 Repeatability and Reproducibility

A repeatability and reproducibility study were performed across lots, operators, days and instruments for the ***PlexPCR***® Flu/RSV/SARS-CoV-2 test, using panels prepared in a simulated matrix. The simulated matrix was prepared using pooled negative

clinical nasopharyngeal swabs collected in VTM diluted with Neat VTM in 1:1 ratio. The panels consisted of one representative strain for each assay target organism spiked into the simulated matrix to a final concentration of 5x LOD, 3x LOD and 1x LOD (**Table 11**).

Testing was performed with two different lots of ***PlexPCR***® Flu/RSV/SARS-CoV-2 mix. Panels were tested by two operators, over ten days, using two different Pilot Lots of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay on two instruments and at three concentrations (5x LOD, 3x LOD, and 1x LOD) per target. In every run, three replicates from each set of panel members were tested.

Between-lot, between-day, between-instrument, between-operator repeatability, and total reproducibility was assessed. Percent agreement and percent coefficient of variation (%CV) was calculated for each panel member based on the expected result and cycle quantification (Cq) in the respective target channel of the assay. Results of repeatability and reproducibility testing are shown in **Table 12 –Table 18.**

Table 11. Concentrations of positive reference material used for repeatability and reproducibility positive panel members.

Positive reference material	1x LOD*	3x LOD*	5x LOD*
FluA	1.6	4.8	8.0
FluB	0.1	0.3	0.5
RSVA	0.3	1.0	1.7
RSVB	0.3	1.0	1.7
SARS-CoV-2	176.0	528.0	880.0
Negative	NA	NA	NA

*Concentration measured in TCID50/mL for FluA, FluB RSVA, and RSVB. Concentration measured in copies/mL for SARS-CoV-2.

Table 12. Repeatability/Reproducibility of the SARS-CoV-2 detection component of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay

Panel member	Total variability			Between Day		Between Operator		Between Lot		Between Instruments		n/N^	Hit rate (%)
	Mean Cq	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV		
SARS-CoV-2 Positive (High)	20.75	1.29	6.2	0.35	1.7	0.12	0.6	0.22	1.1	0.26	1.3	90/90	100
SARS-CoV-2 Positive (Medium)	21.47	0.56	2.5	0.52	2.4	0.23	1.1	0.16	0.7	0.06	0.3	90/90	100
SARS-CoV-2 Positive (Low)	23.13	1.13	4.9	0.36	1.5	0.17	0.8	0.03	0.1	0.22	1.0	90/90	100

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 13. Repeatability/Reproducibility of the FluA detection component of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay													
Panel member	**Total variability**			**Between Day**		**Between Operator**		**Between Lot**		**Between Instruments**		**n/N^**	**Hit rate (%)**
	Mean Cq	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**		
FluA Positive (High)	22.34	0.76	3.3	0.4	1.8	0.27	1.2	0.05	0.2	0.12	0.5	90/90	100
FluA Positive (Medium)	23.27	1.42	6.1	0.36	1.5	0.39	1.7	0.22	1.0	0.37	1.6	90/90	100
FluA Positive (Low)	24.71	1.07	4.3	0.55	2.2	0.1	0.4	0.11	0.4	0.39	1.6	88/90	97.7

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 14. Repeatability/Reproducibility of the FluB detection component of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay													
Panel member	**Total variability**			**Between Day**		**Between Operator**		**Between Lot**		**Between Instruments**		**n/N^**	**Hit rate (%)**
	Mean Cq	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**		
FluB Positive (High)	23.18	0.82	3.5	0.69	3.0	0.44	1.9	0.11	0.5	0.07	0.3	90/90	100
FluB Positive (Medium)	23.76	0.73	3.1	0.53	2.3	0.37	1.6	0.04	0.2	0.06	0.3	90/90	100
FluB Positive (Low)	25.16	0.86	3.4	0.83	3.3	0.4	1.6	0.17	0.7	0.16	0.6	88/90	97.7

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 15. Repeatability/Reproducibility of the RSVA detection component of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay													
Panel member	**Total variability**			**Between Day**		**Between Operator**		**Between Lot**		**Between Instruments**		**n/N^**	**Hit rate (%)**
	Mean Cq	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**		
RSVA Positive (High)	23.54	0.92	3.9	0.54	2.2	0.48	2.1	0.19	0.8	0.25	1.1	90/90	100
RSVA Positive (Medium)	24.46	1.15	4.7	0.83	3.4	0.39	1.6	0.15	0.6	0.08	0.3	90/90	100
RSVA Positive (Low)	27.33	2.5	9.1	2.45	8.4	1.35	4.9	0.69	2.5	0.72	2.6	89/90	99

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 16. Repeatability/Reproducibility of the RSVB detection component of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay													
Panel member	**Total variability**			**Between Day**		**Between Operator**		**Between Lot**		**Between Instruments**		**n/N^**	**Hit rate (%)**
	Mean Cq	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**		
RSVB Positive (High)	24.06	0.8	3.3	0.41	1.7	0.15	0.6	0.23	0.9	0.3	1.3	90/90	100
RSVB Positive (Medium)	27.74	1.13	4.5	0.49	1.9	0.35	1.4	0.14	0.5	0.28	1.1	90/90	100
RSVB Positive (Low)	25.24	2.57	9.3	0.62	2.3	0.54	1.9	1.33	4.8	0.74	2.7	89/90	99

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 17. Repeatability/Reproducibility for between run variability for all targets of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay					
Panel member	**Mean Cq**	**SD**	**%CV**	**n/N^**	**Hit rate (%)**
SARS-CoV-2 Positive (High)	21.13	0.23	1.1	6/6	100
SARS-CoV-2 Positive (Medium)	22.39	0.19	0.9	6/6	100
SARS-CoV-2 Positive (Low)	23.19	0.41	1.8	6/6	100
FluA Positive (High)	23.01	0.17	0.7	6/6	100
FluA Positive (Medium)	23.54	0.19	0.8	6/6	100
FluA Positive (Low)	24.96	1.20	4.8	6/6	100
FluB Positive (High)	24.11	0.25	1.0	6/6	100
FluB Positive (Medium)	24.00	0.43	1.8	6/6	100
FluB Positive (Low)	25.72	0.60	2.3	6/6	100
RSVA Positive (High)	24.10	0.14	0.6	6/6	100
RSVA Positive (Medium)	24.69	0.52	2.1	6/6	100
RSVA Positive (Low)	29.66	0.01	0.0	6/6	100
RSVB Positive (High)	24.28	0.18	0.8	6/6	100
RSVB Positive (Medium)	25.37	0.37	1.5	6/6	100
RSVB Positive (Low)	27.88	0.39	1.4	6/6	100

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

Table 18. Repeatability/Reproducibility for within run variability for all targets of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay					
Panel member	**Mean Cq**	**SD**	**%CV**	**n/N^**	**Hit rate (%)**
SARS-CoV-2 Positive (High)	20.49	0.2	1.0	3/3	100
SARS-CoV-2 Positive (Medium)	21.15	0.1	0.5	3/3	100
SARS-CoV-2 Positive (Low)	22.39	0.2	0.9	3/3	100
FluA Positive (High)	22.09	0.4	1.8	3/3	100
FluA Positive (Medium)	22.81	0.2	0.9	3/3	100
FluA Positive (Low)	24.61	0.2	0.8	3/3	100
FluB Positive (High)	23.04	0.3	1.3	3/3	100
FluB Positive (Medium)	23.96	0.0	0.0	3/3	100
FluB Positive (Low)	25.86	0.5	1.9	3/3	100
RSVA Positive (High)	23.15	0.5	2.2	3/3	100
RSVA Positive (Medium)	24.28	0.2	0.8	3/3	100
RSVA Positive (Low)	27.17	1.1	4.0	3/3	100
RSVB Positive (High)	24.18	0.1	0.4	3/3	100
RSVB Positive (Medium)	26.29	0.4	1.5	3/3	100
RSVB Positive (Low)	29.53	1.0	3.4	3/3	100

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

16.2.2 Analytical sensitivity

The limit of detection (LOD) of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay was determined using representative strains of each assay target strains diluted in a negative matrix. Preliminary LOD concentrations were determined by performing a five-point, two-fold dilution series of each virus strain tested. At least three replicates of each of the dilutions were tested to determine a preliminary LOD using one lot of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay. The LOD was defined as the lowest concentration that had a positivity rate of ≥ 95%.

The LOD concentrations determined in the preliminary study were confirmed by testing 20 replicates of the same virus strain diluted to the preliminary LOD concentrations. The final confirmed LODs for each of the seven virus strains are presented in **Table 19** below.

Table 19. Summary table for confirmed LOD concentrations for all assays of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.

Target organism	Strain	Confirmed LOD	n/N^	Hit Rate
FluA (H1N1)	California/07/09	1.6 $TCID_{50}$/mL	20/20	100
FluA (H3N2)	Victoria/361/11	6.4 $TCID_{50}$/mL	20/20	100
FluB	Malaysia/2506/04	0.1 $TCID_{50}$/mL	20/20	100
FluB	Washington/02/19	0.5 $TCID_{50}$/mL	20/20	100
RSVA	3/2015 Isolate #3	0.3 $TCID_{50}$/mL	20/20	100
RSVB	3/2015 Isolate #1	0.3 $TCID_{50}$/mL	20/20	100
SARS-CoV-2	USA-WA1/2020	176.0 copies/mL	20/20	100

^Number of correctly identified replicates (n) over the total number of replicates tested (N)

The analytical sensitivity of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay was also evaluated using the Second WHO International Standard for SARS-CoV-2 RNA NIBSC code: 22/252. A dilution series with multiple concentrations of the Second WHO IS SARS-CoV-2 (22/252) was tested with each concentration tested in replicates of 3. This dilution series testing established the lowest concentration which gave a 100% hit rate (3/3 replicates correctly detected). Five borderline concentrations around the concentration that gave a 100% hit rate were defined and used to establish the limit of detection using the Probit analysis. The LoD with the Second WHO IS SARS-CoV-2 (22/252) standard, defined as the lowest concentration that passed at 95% hit rate was determined to be 108 IU/mL (95%: 82-171).

16.2.3 Inclusivity

In silico analysis

In silico analysis of polymorphisms in oligonucleotide binding sites was performed to evaluate the inclusivity of the assay primers and probes included in the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay. Sequences with isolation dates between 2015-2022 (for influenza and RSV), and post-January 2022 for SARS-CoV-2, were downloaded from GISAID. Oligos were only assessed against designed targets. All assays are predicted to amplify and detect the required targets. *In silico* monitoring is continuously ongoing to ensure continued inclusivity to current strains and reported variants. Please contact tech@speedx.com.au for more information.

Wet Lab testing Inclusivity testing of the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay was performed to demonstrate the detection of additional representative strains of the assay target organisms. All additional strains were tested at three-fold or five-fold the confirmed LOD for the given assay target, in triplicate. Below are the summarised results of inclusivity testing for each assay target (**Table 20**).

Table 20. List of inclusivity organisms tested in wet lab and the hit rate of correctly detected strains.			
Target organism	**Strain**	**Concentrations tested**	**Hit rate (%)**
FluA (H1N1)	A/Swine/Canada/6294/09	3xLOD	100
FluA (H1N1)	A/NY/02/2009	3xLOD	100
FluA (H1N1)	A/Mexico/4108/2009	3xLOD	100
FluA (H1N1)	NY/01/09	3xLOD	100
FluA (H1N1)	NY/03/09	3xLOD	100
FluA (H1N1)	PR/8/34	3xLOD	100
FluA (H1N1)	A/Victoria/4897/2022	3xLOD	100
FluA (H3N2)	A/Thailand/8/2022	3xLOD	100
FluA (H3N2)	Texas/50/12	3xLOD	100
FluA (H3N2)	Wisconsin/67/05	3xLOD	100
FluA (H3N2)	Brisbane/10/07	3xLOD	100
FluA (H3N2)	Hong Kong/8/68	3xLOD	100
FluB	Lee/40	3xLOD	100
FluB	Panama/45/90 (Victoria lineage)	5xLOD	100
FluB	Florida/02/06 (Victoria lineage)	5xLOD	100
FluB	Massachusetts/2/12 (Yamagata lineage)	3xLOD	100
FluB	Wisconsin/1/10 (Yamagata lineage)	3xLOD	100
FluB	Texas/6/11 (Yamagata lineage)	5xLOD	100
FluB	Brisbane/33/08 (Victoria lineage)	3xLOD	100
FluB	Brisbane/60/08 (Victoria lineage)	3xLOD	100
FluB	Florida/04/06 (Yamagata lineage)	3xLOD	100
FluB	Florida/07/04 (Yamagata lineage)	5xLOD	100
FluB	B/Austria/1359417/2021	5xLOD	100
RSVA	2014 Isolate 342	3xLOD	100
RSVA	2014 Isolate 341	3xLOD	100
RSVA	12/2014 Isolate #12	3xLOD	100
RSVB	CH93(18)-18	3xLOD	100
RSVB	11/2014 Isolate #2	3xLOD	100
RSVB	12/2014 Isolate #1	3xLOD	100
SARS-CoV-2 (Alpha; B.1.1.7)	England/204820464/2020	3xLOD	100
SARS-CoV-2 (Beta; B.1.351)	South Africa/KRISP-K005325/2020	3xLOD	100
SARS-CoV-2 (Gamma; P.1)	Japan/TY7-503/2021	3xLOD	100
SARS-CoV-2 (Delta; B.1.617.2)	USA/PHC658/2021	3xLOD	100
SARS-CoV-2 (Omicron; B.1.1.529)	hCoV-19/USA/MD-HP20874/2021	3xLOD	100

16.2.4 Analytical Specificity

In silico analysis

In silico analysis was performed to evaluate the potential for cross-reactivity of oligos included in the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 assay with genome sequences from bacterial and fungal species identified in the Respiratory Microbiome analysis, non-target viruses, and the human genome. Potential oligonucleotide binding sites that could form potential PCR amplicons were identified. Amplicon sequences were then analysed for potential ***Plex****Zyme*® binding sites. Results of *in silico* analysis indicate no non-target organisms are predicted to be detected by these assays.

Wet lab testing

A panel of 28 microorganisms including organisms commonly found in the human respiratory tract, as well as those closely related to assay targets were evaluated for evidence of cross-reactivity in the ***Plex****PCR*® Flu/RSV/SARS-CoV-2 assay. A list of organisms tested is shown in **Table 21.** Non-target organisms were spiked into negative reference material at 10^5 units/mL for viruses, and at 10^6 units/mL for bacteria and fungus.

Testing was performed in triplicate in the absence of the assay targets. No positive signals were generated in the ***Plex****PCR*® SARS-CoV-2 assay in any of these experiments in the absence of target and there was no impact observed on the performance of the assay due to the presence of high concentrations of any microorganism tested.

Table 21. Representative strains of non-target organisms and tested concentrations used for verification of assay target exclusivity of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.

Non-target organism	Strain	Organism type	Concentration
Achromobacter denitrificans	Z062	Bacterium	10^6 CFU/mL
Adenovirus 1	Adenoid 71	Virus	10^5 $TCID_{50}$/mL
Bordetella pertussis	9832	Bacterium	10^6 copies/mL
Candida albicans	14053	Fungus	10^6 CFU/mL
Chlamydia pneumoniae	53592	Bacterium	10^6 IFU/mL
Enterovirus A71	BrCr	Virus	10^5 $TCID_{50}$/mL
Haemophilus influenzae	49144	Bacterium	10^6 copies/mL
Human coronavirus	229E	Virus	10^5 copies/mL
Human coronavirus^^	NL63	Virus	10^5 copies/mL
Human coronavirus	OC43	Virus	10^5 copies/mL
Human metapneumovirus^	Peru6-2003	Virus	10^5 copies/mL
Human parainfluenza virus 1	N/A	Virus	10^5 $TCID_{50}$/mL
Human parainfluenza virus 2	1/2015 Isolate #2	Virus	10^5 $TCID_{50}$/mL
Human parainfluenza virus 3	4/2015 Isolate #2	Virus	10^5 $TCID_{50}$/mL
Human parainfluenza virus 4	N/A	Virus	10^5 $TCID_{50}$/mL
Legionella pneumophila	Philadelphiae 33152	Bacterium	10^6 genomes/mL
MERS-coronavirus^^	Florida/USA-2 Saudi Arabia 2014	Virus	10^5 copies/mL
Mycoplasma pneumoniae	M129	Bacterium	10^6 CCU/mL
Neisseria flavescens	17913	Bacterium	10^6 genomes/mL
Neisseria subflava	23930	Bacterium	10^6 genomes/mL

Table 21. Representative strains of non-target organisms and tested concentrations used for verification of assay target exclusivity of the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.			
Non-target organism	**Strain**	**Organism type**	**Concentration**
Pooled human nasal wash	-	Saline wash	-
Pseudomonas aeruginosa	27853	Bacterium	10^6 genomes/mL
Rhinovirus 17	33342	Virus	10^5 $TCID_{50}$/mL
SARS-CoV-1^^	2003-00592	Virus	10^5 copies/mL
Staphylococcus epidermis	14990	Bacterium	10^6 CFU/mL
Streptococcus pneumoniae	49619	Bacterium	10^6 genomes/mL
Streptococcus pyogenes	49399	Bacterium	10^6 CFU/mL
Streptococcus salivarius	25975	Bacterium	10^6 genomes/mL
Cytomegalovirus	MBC-016	Virus	10^5 copies/ mL
Epstein-Barr Virus	P3HR-1	Virus	10^5 copies/ mL

^ Non-target organism unavailable. Commercially available quantified extracted whole genome spiked into artificial background tested in place of organism spiked into negative reference material.
^^ High concentration non-target organism unavailable. Commercially available non-target organism extracted using QIAamp MiniElute Virus Spin Kit (QIAGEN) for subsequent spiking into artificial background and testing with the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay.

16.2.5 Competitive Inhibition

The competitive inhibition between assay targets was verified by testing combinations of high concentration (1000x LOD) and low concentration (3x LOD) positive reference material contrived using the assay target organisms as listed in **Table 22.** As the assay target organisms RSVA and RSVB are detected in the same target channel (Cy5), RSVA and RSVB were not combined in the same tested sample. Each combination was tested with the ***PlexPCR***® Flu/RSV/SARS-CoV-2 assay in triplicate. Additionally, each assay target organism was tested at both the respective high concentration and low concentration when in the absence of any other assay target organism, in triplicate. The assay was able to correctly detect all the targets and no impact of competitive inhibition on the assay performance was observed (**Table 23**).

Table 22. Summary of assay target organism and concentration combinations tested using the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.						
Assay target organism concentration*					**n/N****	**Competitive inhibition**
FluA	**FluB**	**RSVA**	**RSVB**	**SARS-CoV-2**		
1000x	3x	3x	-	3x	3/3	**PASS**
1000x	3x	-	3x	3x	3/3	**PASS**
3x	1000x	3x	-	3x	3/3	**PASS**
3x	1000x	-	3x	3x	3/3	**PASS**
3x	3x	1000x	-	3x	3/3	**PASS**
3x	3x	-	1000x	3x	3/3	**PASS**
3x	3x	3x	-	1000x	3/3	**PASS**
3x	3x	-	3x	1000x	3/3	**PASS**

* Fold-increase of assay target LOD

** Number of correctly identified replicates (n)/Total number of replicates (N)

16.2.6 Potential interfering substances

Potentially interfering exogenous (**Table 23**) and endogenous (**Table 24**) substances that might be present in respiratory specimens were assessed for their impact on the performance on the ***PlexPCR***® Flu/RSV/SARS-CoV2 assay. All substances were tested in the presence and absence of any assay target organism in triplicate. There was no evidence of a negative impact on assay performance when contrived samples containing the potential interferents at the indicated concentrations were tested.

Table 23. List of exogenous potential interferents, and concentration, tested with the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.

Product name/substance	Active ingredient	Test concentration of substance
Difflam® *plus* anaesthetic sore throat lozenges (herein lozenges)	Benzydamine Lidocaine Dichlorobenzyl alcohol	3 mg/mL
Difflam® *forte* sore throat spray (herein throat spray)	Benzydamine	5% (v/v)
Robitussin® cough syrup (herein cough syrup)	Guaifenesin Dextromethorphan hydrobromide monohydrate	5% (v/v)
Nicorette® QuickMist Nicotine spray (herein QuickMist)	Nicotine	0.03 mg/mL
Oxymetazoline	Oxymetazoline	1 mg/mL
Benzocaine	Benzocaine	1 mg/mL
Bactroban 2% Cream	Mupirocin	5 mg/mL
Oseltamivir Lupin 75mg capsule	Oseltamivir	7.5 mg/mL
Tobramycin Viatris	Tobramycin	4 ug/mL

Table 24. List of endogenous potential interferents, and concentration, tested with the *PlexPCR*® Flu/RSV/SARS-CoV-2 assay.

Substance	Test concentration of substance
Mucin (Bovine; Submaxillary gland) (herein mucin)	2.5 mg/mL
Whole blood (Human; Single donor) (herein whole blood)	5% (v/v)

16.3 Summary of Safety and Performance (SSP)

Summary of Safety and Performance of the device will be available on the EUDAMED as soon as the EUDAMED is fully operational.

This information can be found in https://ec.europa.eu/tools/eudamed.

17 Customer and Technical support

Please contact Technical Support for questions on reaction setup, cycling conditions and other enquiries.

Tel: +61 2 9209 4169, Email: tech@speedx.com.au

18 References

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19 Glossary

European Conformity
For *In Vitro* Diagnostic Use

Catalogue number

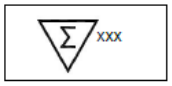
Batch code

Authorised Representative
In the European Community

Manufacturer

Date of manufacture

Temperature limit



Contains sufficient for
xxx determinations

Use by Date

Importer

Keep away from sunlight

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