AmphiSense Gold Respiratory Kit 1 Instructions for Use

ASP5-CFX-200

ASP5-CFX-1000

IFU-10284(01)
English

For In Vitro Diagnostic Use (IVD)
For Prescription Use Only

AmphiSense Gold Respiratory Kit 1

Real-Time PCR test for the detection of Influenza A, Influenza B, Respiratory Syncytial Virus and SARS-CoV-2 for use with:

Specimen Types	Extraction Platforms	PCR Platforms
Nasopharyngeal Specimens	Thermo Scientific™ KingFisher™ Flex Extraction System	Bio-Rad CFX96 Dx System

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Intended Use

The AmphiSense Gold Respiratory Kit 1 is a multiplexed real-time RT-PCR test intended for the qualitative detection and differentiation of RNA from SARS-CoV-2, influenza A, influenza B, and respiratory syncytial virus (RSV) in nasopharyngeal (NP) swab specimens collected from individuals exhibiting signs and symptoms of respiratory tract infection who are suspected of COVID-19, influenza A, influenza B, or RSV infection.

Results are for the identification of SARS-CoV-2 RNA, Influenza A RNA, Influenza B RNA, and RSV RNA. RNA from SARS-CoV-2, Influenza A, Influenza B, and RSV is generally detectable in nasopharyngeal swabs during the acute phase of infection. Positive results are indicative of active infection. Laboratories within the United States and its territories are required to report all SARS-CoV-2 results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 or Influenza A/B or RSV 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.

Positive results do not rule out co-infection with other non-panel viruses or bacterial infection. The virus detected may not be the definitive cause of illness. The use of additional laboratory tests and clinical presentation must be considered to diagnose respiratory viral infection.

The AmphiSense Gold Respiratory Kit 1 may not detect emerging novel strains of Influenza A. If infection with a novel influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions for novel virulent influenza viruses and sent to a state or local health department for testing. Viral culture should not be performed in these cases unless a BSL 3+ facility is available for use.

Principles of Procedure

The nucleic acids are isolated and purified from nasopharyngeal swabs using a previously FDA cleared nucleic acid extraction system. The extracted viral RNA is reverse transcribed into cDNA with the the AmphiSense Gold Respiratory Kit 1. Probes present in the master mix anneal to a specific target sequence 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, causing the reporter fluorophore to separate from the quencher fluorophore, generating a fluorescent signal. With each cycle, additional reporter fluorophore molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at specific PCR cycles.

Components and Storage

Kit Components

Table 1: *Kit Components included with the AmphiSense Gold Respiratory Kit 1*

Component	Description	200 Reaction Kit	1000 Reaction Kit	Storage Conditions
MP-ASP5-CFX-PC	2X Multiplex Plus HotStart MasterMix	2 × 1 mL	10 × 1 mL	-20°C
RTM-ASP5-CFX-PC	RT Mix	1 × 250µL	5 × 250µL	-20°C
CFR7PPM-ASP5-CFX-PC	CoVi,FluA/B,RSV Plus 5 Primer Probe Mix	2 × 100µL	10 × 100µL	-20°C
NFW-ASP5-CFX-PC	Nuclease Free Water	2 × 350µL	10 × 350µL	-20°C

Table 2: *Reagents and kits required but not provided with the AmphiSense Gold Respiratory Kit 1*

Name	Manufacturer	Part Number
Molecular Grade Water (Not DEPC Treated)	Any	Any
Molecular grade 100% Ethanol	Any	Any
MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit	Applied Biosystems™	A48383

Controls Required but Not Provided with the Kit

External positive and negative controls are required to be included in every run to accurately interpret patient test results. Positive and Negative Controls should be extracted along with patient samples and included at least once per PCR plate. An appropriate Negative Control will be negative for all targets including the Endogenous Internal Control (RNase P). An appropriate Positive Control will be positive for Influenza A, Influenza B, RSV, SARS-CoV-2 and the endogenous Internal Control (RNase P). Users may purchase and qualify their own IVD external Positive and Negative Controls or purchase the options below.

Table 3: *Commercially Available External Positive and Negative Controls*

Name	Manufacturer	Part Number
SARS-CoV-2, Flu, RSV Positive Run Control	Exact Diagnostics	COVFLU
Respiratory Negative Swab (REDx™FLOQ®)	Microbix Biosystems	RED-S-99-01

Consumable Items Required but Not Provided with The Kit

Table 4: *Consumable items required but not provided with the AmphiSense Gold Respiratory Kit 1*

Name	Manufacturer	Part Number or Description
Hard-Shell 96-Well PCR Plates, low profile, thin wall, white/white, or equivalent	Bio-Rad	barcoded (PN HSP9655), non barcoded (PN HSP9955), or unskirted (PN MLL9651)
Microseal 'B' PCR Plate Optical Sealing Film, or equivalent	Bio-Rad	MSB1001
Single-Channel Pipette	Any	Pipettes capable of measuring in the following ranges: 0.2-10 µL, 2-20 µL, 20-200 µL, & 100-1000 µL
Multi-Channel Pipette	Any	Pipettes capable of measuring in the following ranges: 0.2-10 µL, 2-20 µL, 20-200 µL, & 100-1000 µL
Sterile, Aerosol Barrier Pipette Tips	Any	Pipette tips for use with single or multichannel pipettes above
MicroCentrifuge tube, 1.5 mL, PCR grade	Any	N/A
2°C-8°C Cold block for 96 well PCR plate	Any	N/A
2°C-8°C Cold Block for 2 mL tubes	Any	N/A
Adhesive PCR plate foil cover	Any	N/A
KingFisher 96 deep-well plate	Thermo Scientific™	95040460, 95040450
KingFisher 96 tip comb for deep-well magnets	Thermo Scientific™	97002534
15 mL Sterile, DNase, RNase free conical screw top tubes	Any	N/A
50 mL Sterile, DNase, RNase free conical screw top tubes	Any	N/A
Disposable Reagent Reservoirs 25 mL, 50 mL, or 100 mL	Any	N/A

Equipment/Instrumentation Required but not supplied

Table 5: *Equipment and instrumentation required but not supplied with the AmphiSense Gold Respiratory Kit 1*

Name	Manufacturer	Part Number
BioRad-CFX96 Dx Real Time Detection System	Bio-Rad	• C1000 Dx Thermal Cycler #1841000-IVD • CFX96 Dx ORM #1845097-IVD (includes CFX Manager Dx Software v3.1 #12007917)
PCR Plate Centrifuge	Any	N/A
Mini-centrifuge to spin down 1.5 and 2 mL tubes	Any	N/A
Vortex	Any	N/A
KingFisher™ Flex Purification System, KingFisher with 96 Deep-well Head	Thermo Scientific™	5400630

Reagent Storage, Handling, and Stability

- All components of the kit must be stored at the appropriate storage conditions as listed in the section *Kit Components*.
- The AmphiSense Gold Respiratory Kit 1 Primer/Probe reagent should be stored at -20°C and protected from light.
- Do not use kit components after the expiration date printed on the label.
- If there is damage to the packaging inside and outside or kit contents have been tampered with or storage condition failed to meet the -20°C condition do not use.
- All reagents, kits, and consumables required but not provided should be stored according to manufacturer labeling.
- AmphiSense Gold Respiratory Kit 1 reagents may be thawed and re-frozen up to three times prior to use
- AmphiSense Gold Respiratory Kit 1 reagents may be thawed and stored at 2-8°C for up to seven (7) days prior to use.
- Dispose of unused reagent and waste in accordance with country, federal, state, and local regulations.

Warnings and Precautions

All procedures should be performed in a laboratory of adequate Bio Safety Level (BSL) as recommended by the CDC and specimens handled within a Biological Safety Cabinet (BSC). Specimens should always be considered potentially infectious and handled in accordance with safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus disease 2019 (COVID-19). https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

Separate work areas should be used for:

- **Reagent Preparation** (e.g., preparation of RT-PCR master mix): No amplified reactions, target solutions, control materials or clinical specimens should be brought into this area. After working in this area, laboratory coat and gloves should be changed before moving into nucleic acid addition area.
- **Specimen preparation/Nucleic acid addition:** Patient specimens should either be extracted and added to the PCR plate in this area.
- **Instrumentation/ Post- PCR** (e.g. BioRad CFX96 Dx): Post amplification PCR plates should be disposed of in this area and not be brought into any Pre-PCR area.
- All reagent, specimen handling, and instrumentation areas must be cleaned with 10% bleach or other similar decontamination product to ensure amplicon and/or specimen contamination risk is minimized. Note: Bleach is not compatible with KingFisher Flex reagents and may cause toxic gas if combined. Do not allow bleach to come in contact with these reagents.

General Handling:

Proper molecular biological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear powder-free latex, vinyl, or nitrile gloves when handling reagent tubes and RNA specimens to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed. During the procedure, work quickly and keep everything at 2°C-8°C on ice or cold blocks, when possible, to avoid degradation of RNA by endogenous or residual RNase.
Clean working surfaces, pipettes, with cleaning reagents that destroy nucleic acids and RNase. To eliminate accelerated deterioration of any plastics and metals, wipe down surfaces with 70% ethanol.
As with any testing procedure, good laboratory practices are essential to the proper performance of this assay.

- All human-sourced materials should be considered potentially infectious and should be handled with universal precautions. If spillage occurs, immediately disinfect with freshly prepared solution of 0.5% sodium hypochlorite (10% v/v bleach). Dispose of cleaning materials in a biohazard waste container.
- Proper personal protective equipment including lab coats, gowns, gloves, eye protection, and biological safety cabinet are recommended for manipulation of clinical specimens. Refer to Microbiologic and Biomedical Laboratories (BMBL), 6th Edition- CDC.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19). See https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html
- Specimen processing should be performed in accordance with national biological safety recommendations. See https://www.cdc.gov/labs/BMBL.html
- If infection with a viral pathogen is suspected, based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Process human clinical specimens within a Class II (or higher) biological safety cabinet (BSC).
- Closely follow procedures and guidelines provided to ensure that the test is performed correctly. Any deviation from the procedures and guidelines may affect the test performance.
- Avoid over exposure of the primer-probe mixes to light for optimal fluorescent signal.
- If exposure of biological materials to skin or mucous membranes occurs, immediately wash the area with large amounts of water. Seek medical advice immediately.
- Do not use components beyond the expiration date printed on the kit boxes.
- Reagents supplied are formulated specifically for use with this kit. Make no substitutions to ensure optimal performance of the kit. Do not mix reagents from different lots.
- Return all components to the appropriate storage conditions after preparing working reagents.
- All equipment used for testing must be calibrated and QC checked in accordance with CLIA regulations.

Specimen Storage

Patient specimens must be collected following laboratory guidelines and the transport media manufacturer's instructions .
For use only with Nasopharyngeal (NP) swabs in Universal Transport Media (UTM) /Viral Transport Media (VTM) from the following manufacturers/matrices in Table 6 below.

Table 6: *Collection and transport media for use with AmphiSense Gold Respiratory Kit 1*

UTM/VTM Name
Hardy Viral Transport Medium
Medschenker STM
Copan UTM

- Nasopharyngeal (NP) swab specimens collected in the transport media types above may be stored at 2°C-8°C for up to six (6) days OR at 30°C for up to twelve (12) hours prior to testing with the AmphiSense Gold Respiratory Kit 1.
- Nasopharyngeal (NP) swab specimens collected in Hardy VTM may be frozen and stored at -70°C for up to 14 days prior to testing the AmphiSense Gold Respiratory Kit 1.
- Nasopharyngeal (NP) swab specimens collected in Hardy VTM and stored at -70°C may undergo up to 3 freeze-thaw cycles prior to testing the AmphiSense Gold Respiratory Kit 1.
- Frozen storage is not recommended for nasopharyngeal (NP) swab specimens collected in Medschenker STM or Copan UTM.

Note: Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending specimens.

AmphiSense Gold Respiratory Kit 1 Nucleic Acid Preparation Procedure

RNA Extraction

General guidelines referenced from Thermo Fisher MAN0019746

- Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block. – Failure to use the proper magnetic head and heat block results in lower yields and potential harm to the instrument
- Ensure that the MVP_2Wash_200_Flex program has been downloaded from the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit product page at www.thermofisher.com and loaded onto the instrument.
- Perform all steps at room temperature (15°C –25°C), unless otherwise noted.
- Precipitates can occur if the Binding Solution is stored when room temperature is too cold. If there are precipitates, warm the Binding Solution at 37°C and gently mix to dissolve the precipitates. Avoid creating bubbles.
- Reagent Mix tables are sufficient for a single reaction. To calculate volumes for other reaction numbers, see the per well volume and add at least a 10% overage.
- (Optional): To prevent evaporation and contamination, cover the prepared processing plates with paraffin film or Clear Adhesive Film then store at room temperature for up to 1 hour while you set up the patient specimen plate.
- Note the following for all kit components and 80% ethanol solution: Open reagent bottles, or reagents poured in reagent reservoirs, have been shown to be stable for ≤2 hours at room temperature.

Guidelines for Binding Bead Mix

1. Vortex Binding Beads thoroughly before each use.
2. Ensure that the beads stay fully mixed within the solution during pipetting.
3. Avoid creating bubbles during mixing and aliquoting.
4. Binding/Bead Mix is very viscous so pipet with care to ensure that the correct volume is added to the specimen.

Extraction Procedure

***NOTE- prior to extraction patient specimens are potentially very infectious and should only be opened and handled in an approved BSL2 safety cabinet.**

Prepare Processing Plate

1. Obtain KingFisher Flex program script MVP_2Wash_200_Flex.bdz from Thermo Fisher website and install on KingFisher instrument.

Table 7: *KingFisher Volumes and Plate Positions*

Plate ID	Deck Position	Plate Type	Reagent	Volume Per Well
Wash 1 Plate	2	KingFisher™ 96 Deep-Well Plate	Wash Solution	500 µL
Wash 2 Plate	3		80% Ethanol	500 µL
Elution Plate	4		Elution Buffer	50 µL
Tip Comb Plate	5	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate or equivalent plate		

NOTE – Due to instrument compatibility and required incubation steps on instrument the elution plate must be a full deep-well plate.

2. Using Table 7 above as a guide prepare plates for number of specimens and configuration in 96-well plate.
3. Prepare 1 plate for Wash buffer (included in kit) with 500 µL per well.
4. IMPORTANT! Wash Solution may develop inert white or brown particulates that float in solution. This is not a cause for concern and does not negatively affect performance.
5. Prepare fresh 80% Ethanol from 100% absolute Ethanol and Nuclease-Free Water. –Prepare enough for 0.5 mL per reaction with overage.
6. Prepare 1 plate for 80% ETOH (from fresh prep above) with 500 µL per well.
7. Prepare 1 deep-well plate with 50 µL elution buffer (included in kit) in each well.

Prepare Specimen Plate

1. Prepare Binding Bead Mix for specimens. Prepare fresh each day.
   a. Make enough Binding Beads for a minimum of 10% overage due to potential foaming (make sure to vortex beads to ensure the bead mix is homogenous)
   b. For each specimen add 265 µL of Binding Buffer and 10 µL Binding Beads.
   c. EXAMPLE – for 10 specimens add enough for 11 (10% overage) add 2.92 mL of Binding Buffer and 110 µL of Beads.
   d. Invert to mix. DO NOT VORTEX
   e. Add 275 µL of Binding Bead Mixture to each well of deep-well specimen plate. Pipet slowly to ensure correct volume added since mixture is viscous. (Invert Binding Bead Mix during additions to prevent settling)
2. Add 200 µL of each patient specimen or control (1 positive and 1 negative control, See below Quality Control Section) to 96 deep-well plate with Binding Bead Mix already added. (Add to the top layer and do not push the pipette tip into the Binding Bead Mix)
3. Add 5 µL of Proteinase K to each patient specimen and control well. (Add to the top layer as well)
4. Select MVP_2Wash_200_Flex.bdz program on instrument and push START.
5. Follow instrument prompts to place Tip comb in deep-well 96-well plate, Elution plate, 80% wash plate, Wash buffer plate and specimen plate in designated positions in the table below. Verify that Position A1 is in the proper orientation for all plates on KingFisher Flex deck.

Table 8: *KingFisher deck positions and final volumes for plates*

Plate ID	Deck position	Plate type	Reagent	Volume per well
Patient specimen / bead plate	1	Deep-well	Beads, buffer, Prot K / patient specimen or control	480 µL
Wash 1 Plate	2	Deep-well	Wash Buffer	500 µL
Wash 2 Plate	3	Deep-well	80% Ethanol	500 µL
Elution Plate	4	Deep-well	Elution Solution	50 µL
Tip Comb	5	Place a 96 Deep-well Tip Comb in a depp-well Plate		

6. Push START one more time and allow instrument to perform program.
7. At conclusion of program, the purified nucleic acid in elution plate is ready for immediate use. Specimen plate may be covered with PCR foil plate adhesive cover and transferred to testing location or stored for later use.
8. Purified nucleic acid may be stored prior to testing with the AmphiSense Gold Respiratory Kit 1 for:
   a. up to eight (8) days at 2-8°C
   b. up to ninety-six (96) hours at 30°C
   c. up to thirty-five (35) days at -70°C with up to three (3) freeze-thaw cycles

Quality Control

Patient specimens must be collected according to CDC guidelines.
The following controls are required to be included in every run to accurately interpret patient test results.

Negative Control

A negative control is needed to monitor contamination of equipment and PCR reagents with amplifiable material. The required Negative control is intended to be included in the RNA extraction and then included as a template at least once per RT-PCR plate (i.e. for each RT-PCR run). This control will be negative for all targets.

Positive Control

A positive control is needed to monitor the integrity of reagents, screen for improper assay set up and RT-PCR reagent failure. The required positive control is intended to be included in the RNA extraction step and then included as template at least once per RT-PCR plate (i.e. for each RT-PCR run). This control will be positive for SARS-Cov-2, Flu A, Flu B and RSV. It will also be positive for the internal control RNaseP.

No Template Control

A no template control (NTC) containing PCR grade nuclease-free water is needed to determine if amplicon contamination occurred during the RT-PCR step. The control consists of 5 µL of water (in place of specimen) at least once per plate (i.e. for each RT-PCR run). The NTC is not extracted. The water is added to the PCR plate during set up.

Endogenous Internal Control

An endogenous internal extraction control is used to monitor poor specimen quality, extraction, and PCR processes and reagent failures. The internal control is the amplification of the human RNaseP gene with its own primer and probe set included in the Kit. Amplification of the RNase P internal control is required to confirm a negative result. Failure to detect the endogenous internal control without detection of assay targets indicates a failed test. RNase P detection is not required when an assay target is positive.

RT-PCR Run Setup

BioRad CFX Dx Run Setup

1. In the BioRad CFX Manager Dx Version 3.1 software home screen, select the Startup Wizard under View tab. Enter or confirm instrument as the instrument to be used. Select run type as User-defined.
2. In the Protocol tab, click **Select Existing...** and navigate to the ASGR1 protocol template.
   - If no ASGR1 protocol is available, create new protocol with thermal cycling parameters as listed below (20 µL reaction volume):

Table 9: *RT-PCR run conditions*

Step	Temperature	Time	Number of Cycles
RT Incubation	50°C	15 minutes	1
Enzyme Activation	95°C	2 minutes	1
Amplification	95°C	3 seconds	40
	60°C**	30 seconds	

**collect fluorescence during annealing/extension phase (60°C) step on all 5 channels.

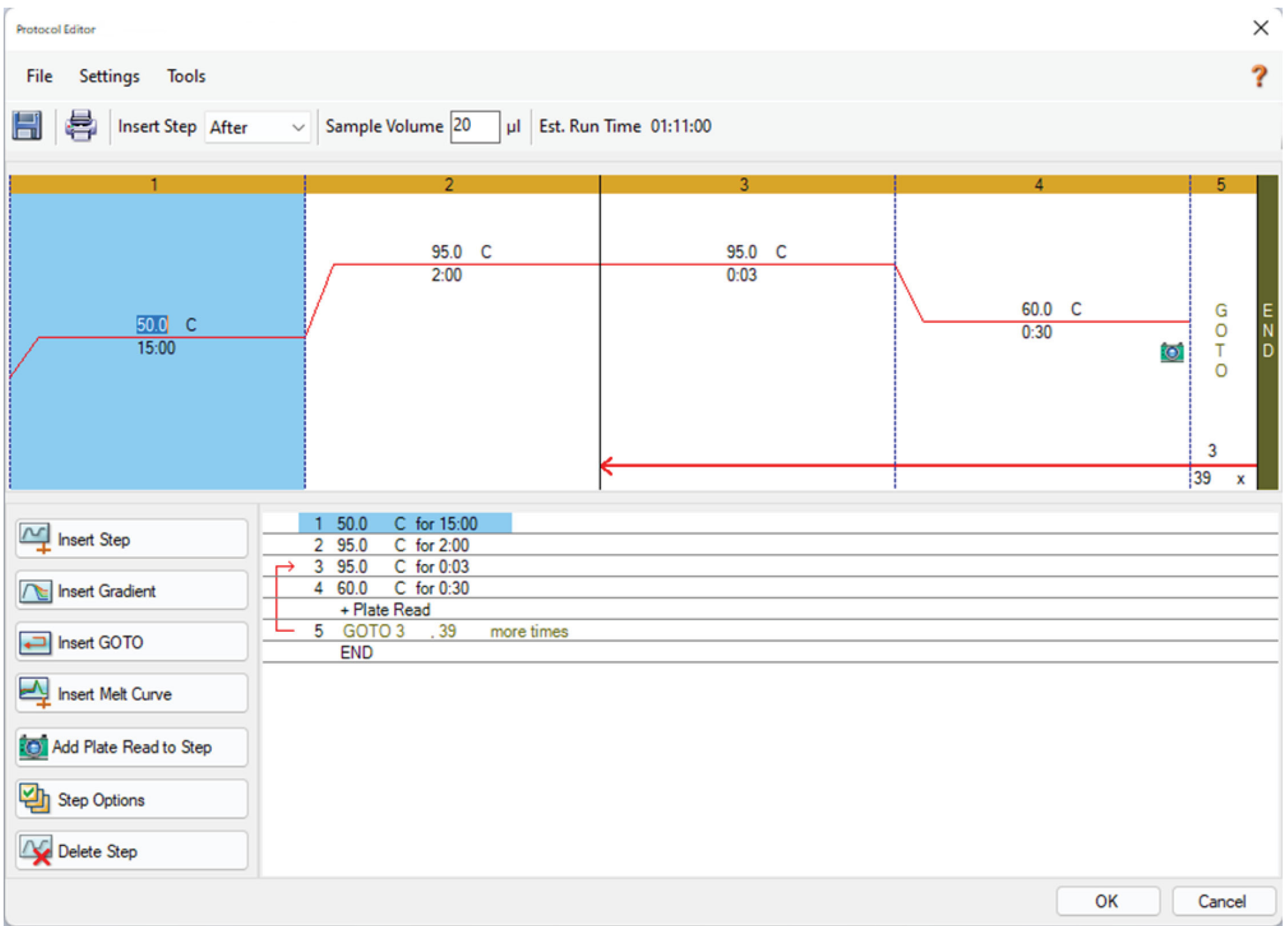


Figure 1: *Cycling parameters*

a. PCR protocol set-up on CFX 96 Dx if no existing protocol found:
    i. Open BioRad CFX Manager Dx software and select user-defined on select run type window.
    ii. In run setup window, click on Edit Selected…
    iii. Adjust cycles as noted in Figure 1, save changes and name template as 'ASGR1'
b. Select "ok" and "next" to proceed to plate set up.

3. In the Plate tab, click Select Existing… and navigate to the ASGR1 template.
    a. For first time use create a template with following steps.
        i. In plate set up (Figure 2) select: "create new" at top. This will bring up the detailed set up panel (Figure 3).
        ii. Set scan mode to all channels.
            1. In select fluorophores check boxes next to all fluorophores in Table 10.
            2. In experiment settings type in the 5 targets from Table 10, "add" each and select OK to proceed.
            3. In settings tab select plate type- "BR White" and plate size – "96 well".
        iii. To select the entire plate, click the small blue box above well A1 in the plate map.
            1. Under Sample Type select "unknown" or control type for each well.
            2. Select wells where controls are located and designate control type.
            3. Select "Load" and "Target Name" and match up the Target and fluorophores from Table 10.
        iv. Select the tab for editing tools and select "Spreadsheet View/Importer".
        v. Export the template to a designated location for later. This template can be used to input patient ID numbers for Import and future use.
4. Input sample setup data.
5. In the Define tab for Targets, confirm that the targets and reporter fluorophores are listed correctly.
6. Confirm all applicable wells Sample Type as Unknown (except for each control). Below are all the targets and fluorophores.

Table 10: *Targets and their fluorophore channels*

Target	Fluorophores
SARS-CoV-2	FAM
Influenza A	Quasar 670
Influenza B	HEX
RNaseP	CalRed 610
RSV	Quasar 705

Verify plate layout has specimens/wells designated for control specimens. Positive control, negative control, and no template control (PCR blank) should be present for each run.

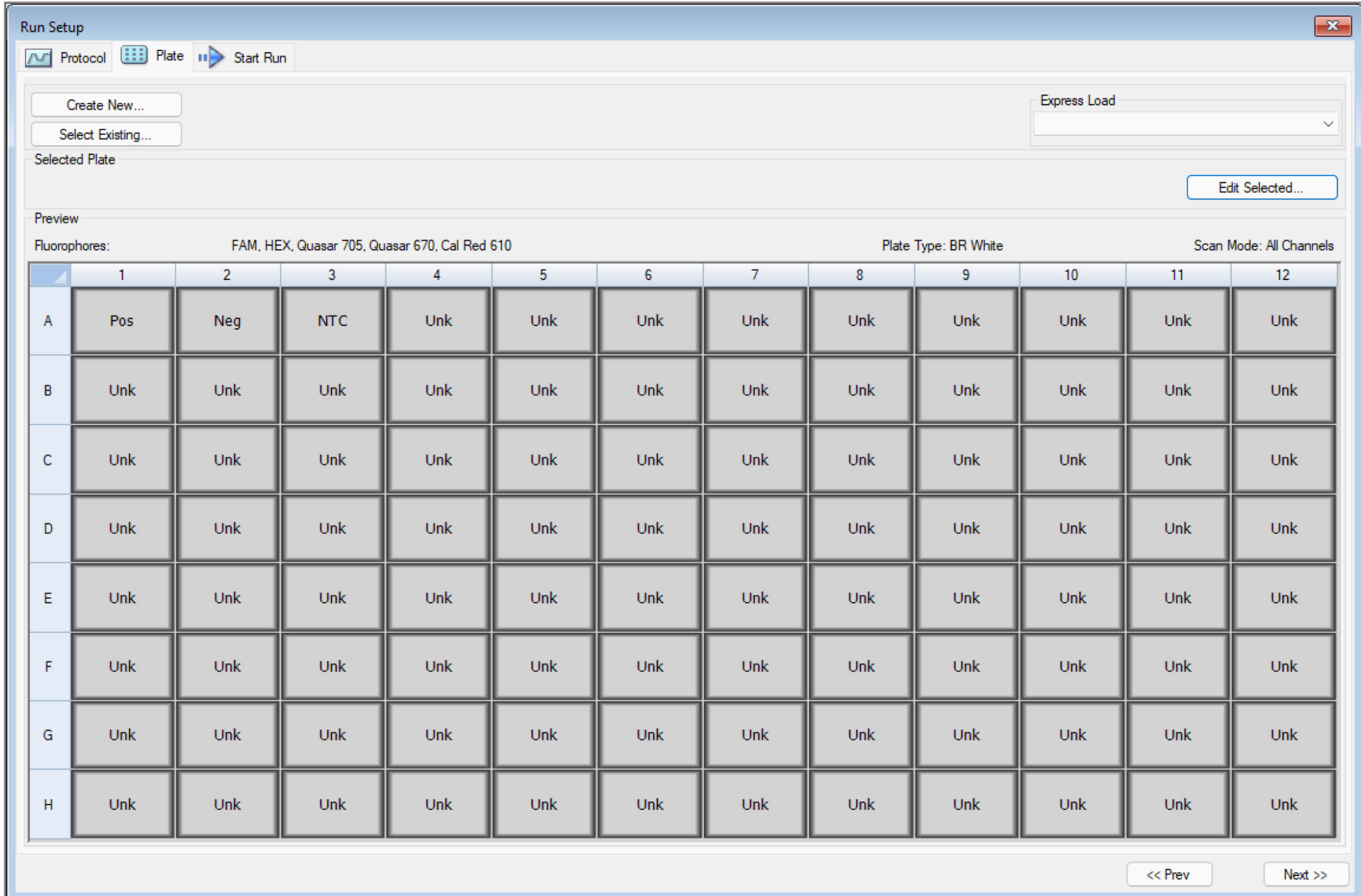


Figure 2: *Run setup*

Plate layout is for example only. Specimens and controls may be located in any plate location or order as compatible with individual lab system or LIS. Proper specimen and control labeling must be maintained and tracked per internal lab protocols.

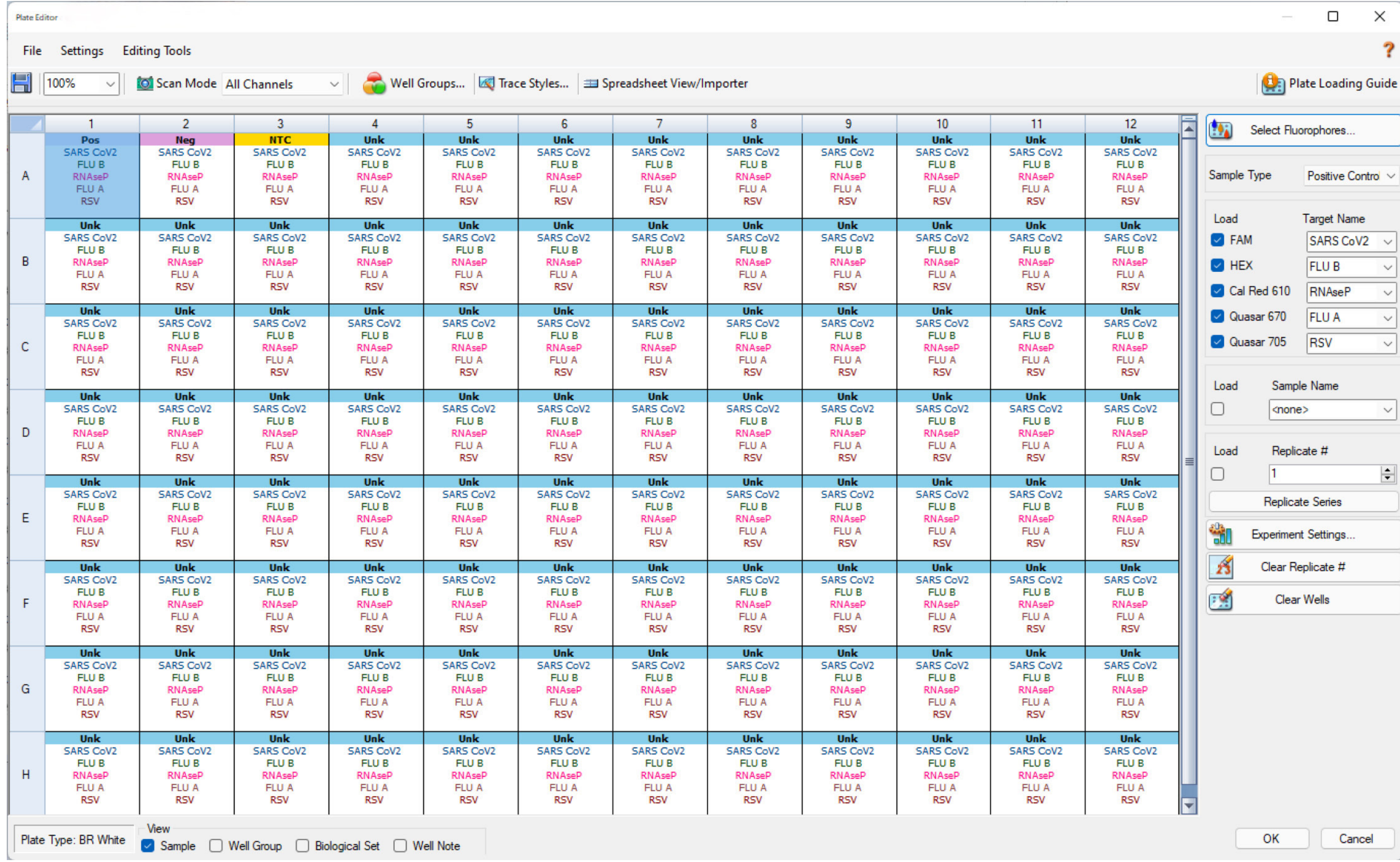


Figure 3: *Detailed plate setup*

RT-PCR Master Mix and Reagent Setup

1. Completely thaw the sterile water, 2X Multiplex PLUS HotStart MasterMix, and CoVi/Flu/RSV Plus 5 Primer/Probe Mix (brown tube) and RT Mix by setting on 2°C-8°C cold block for ~30 minutes. Once thawed, briefly centrifuge to collect at the bottom of the tube.
2. Vortex the reagent tubes at max speed for 3 seconds to mix, then spin down briefly to collect at bottom of tube. The 2X Multiplex PLUS HotStart MasterMix can have sedimentation. If this occurs, vortex until sediment is fully dissolved.
3. Pipette the following components into a 1.5 mL or larger tube, in order shown below, in designated setup area.

Table 11: *AmphiSense Gold Respiratory Kit 1 PCR set-up volumes*

Component	Volume 1x Reaction (µL)	Volume 96 reactions (96-well plate (µL)) including 7% overage
Nuclease-free Water	2.75	283.8
CoVi/Flu/RSV Plus 5 Primer/Probe Mix	1	103.2
2X Multiplex PLUS HotStart MasterMix	10	1032
RT Mix	1.25	129

Note: For quantities less than 96 reactions prepare 1x reaction volume x quantity of tests including controls (3) plus 10% overage. Only make enough master mix for immediate use. Discard any unused master mix after dispensing.

4. Mix the master mix by capping the tube, vortex briefly, and spinning down briefly to collect mix.
5. Pipette 15µL of the master mix to all wells of the 96-well PCR plate kept at 2°C-8°C.
6. Pipette 5µL of specimen, positive control, or negative control to the appropriate wells of the 96-well PCR plate.
7. Pipette 5µL of nuclease-free water into the well designated as no template control (PCR blank).
8. Seal the plate, then spin down in a plate centrifuge at 2000 to 3000 RFG for 30 seconds to collect the reaction mix. *Note: The reaction mix in the sealed plate may be temporarily stored prior to the RT-PCR run: at room temperature (15-25°C) for no more than 25 minutes, OR refrigerated (2-8°C) for no more than five (5) hours.*
9. Place the plate in the BioRad CFX96 Dx instrument. *Note: Once the plate has been placed on the instrument, the RT-PCR run must be started within ten (10) minutes or less.*
10. On the PCR instrument's BioRad CFX Manager Dx software, review the run information that was configured in RT-PCR Run Setup.
11. In the Start Run tab, check the box for the applicable instrument and select Start Run.
12. Enter a file name in the dialog box that prompts you to save the run file, then save the file.
13. RT-PCR run time is approximately 1h:11m.

Interpretation of Results

1. In the BioRad CFX Manager Dx software after the run is complete, select File and Save As and select a location to save the run's .pcrd file.
2. If performing analysis on an external computer use a USB drive or other method, open the run data file from the computer connected to the instrument to the computer that will be performing the analysis.
3. Open the CFX Manager Dx software and open the run data file for the completed run.
4. Use CFX Manager Dx software default settings and make following adjustments for analysis.
5. In the 'Data Analysis' pop-up window under the 'Quantification' tab, select 'Settings' in the main menu then select 'Baseline Setting' and both 'Baseline Subtracted Curve Fit' and 'Apply Florescence Drift Correction'.

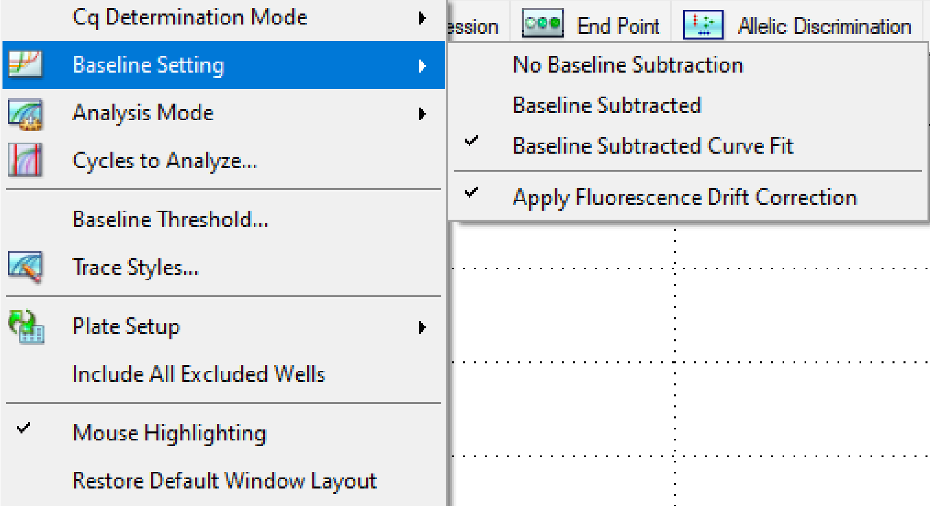
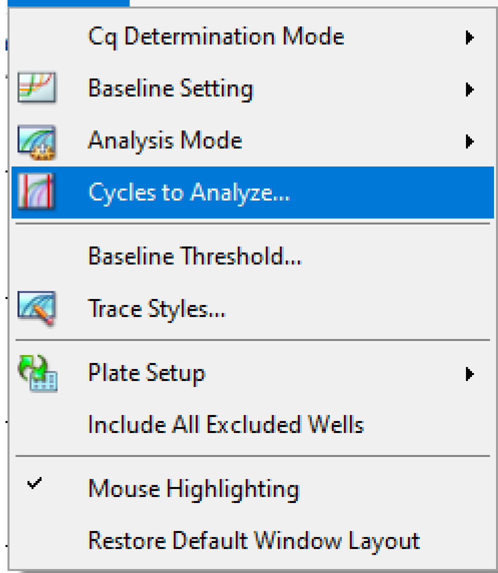


Figure 4: *Baseline settings*

6. Edit the Cycles to Analyze by selecting the Cycles to Analyze under settings and set the lower cycles to 5. This will limit the baseline analysis to cycles 5 to 40 and minimize baseline noise.
7. For Analysis Mode select "Target".



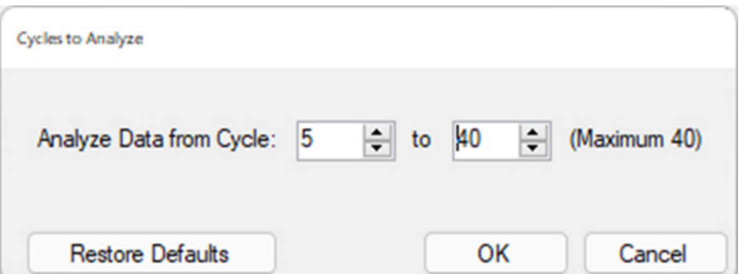


Figure 5: *Cycles to Analyze*

8. Edit the baseline and cycle threshold settings for SARS-CoV-2 by performing the followings steps: Under the ‘Amplification’ curves, uncheck the box on the left of all boxes except ‘FAM’. From the main menu, select ‘Settings’ then select ‘Baseline Threshold…’ to open the ‘Baseline Threshold’ pop-up window. Under the ‘Baseline Cycles’ section, select ‘Auto Calculated’. In the ‘Single Threshold’ section, select ‘User Defined:’ and set this to “500.00”. Click ‘OK’ to proceed.

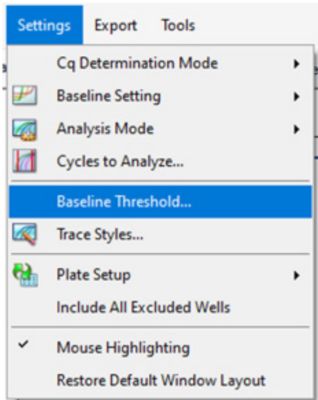


Figure 6: *Baseline Threshold*

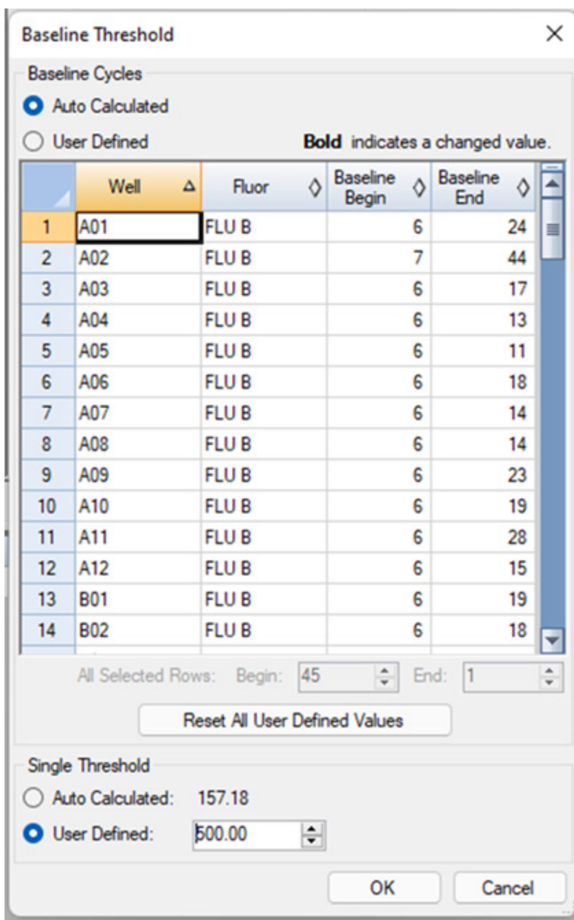


Figure 7: *Manual threshold settings*

Repeat instructions in step 8 for Flu B (HEX), Flu A (Quasar 670), RSV (Quasar 705), and RNaseP (CalRed610) to set each of those to a user defined threshold of 500.00.

Table 12: *RFU cut off for each target*

Target	Fluorophores	User Defined Threshold
SARS-CoV-2	FAM	500
Influenza A	Quasar 670	500
Influenza B	HEX	500
RNaseP	CalRed610	500
RSV	Quasar 705	500

9. After defining baseline and cycle threshold settings for SARS-CoV-2, Flu A, Flu B, RSV, and RNaseP, check all boxes on the left of FAM, Quasar 670, HEX, CalRed610 and Quasar 750 to select all markers.
10. Select the 'Quantification Data' tab. Right-click over the 'Results' table, and chose the desired method for exporting the data (e.g. 'Export to Excel...').
11. Review positive/negative/no template control to ensure their validity before continuing with the run analysis. If controls are not valid, the patient results cannot be interpreted.
12. Positive control must have a Cq value for all viral markers to be valid.
13. Negative control must have an NaA for all targets to be valid.
14. NTC control must have an NaA for all targets to be valid.

Table 13: *Table of control interpretations*

Control	Control Pass	Control Fail	Action if Fail
Negative Control	No Detection of: Influenza A, Influenza B, RSV, SARS-CoV-2, and RNaseP	Detection of ANY: Influenza A, Influenza B, RSV, SARS-CoV-2, or RNaseP	Invalid specimens: Repeat extraction for the specimens in the failed RT-PCR run
Positive Control	Detection of: Influenza A, Influenza B, RSV, SARS-CoV-2, and RNaseP	No Detection of: Influenza A, Influenza B, RSV, SARS-CoV-2, or RNaseP	Invalid specimens: Repeat extraction for the specimens in the failed RT-PCR run
NTC	No Detection of: Influenza A, Influenza B, RSV, SARS-CoV-2, and RNaseP	Detection of ANY: Influenza A, Influenza B, RSV, SARS-CoV-2, or RNaseP	Invalid Run: If NTC result shows a failure repeat RT-PCR run.
Internal control	RNaseP detected in patient specimen	RNaseP not detected in patient specimen unless virus detected.	Repeat specimen where RNaseP not detected

15. All specimens must have a valid internal control and detect RNaseP with a valid Cq value unless a viral positive is detected in the specimen with a valid Cq value.
16. Any specimen showing a Cq value for a viral marker is positive for that analyte and should be reported according to Clinical testing guidelines.
17. Save the report by defining the 'File name' as appropriate to laboratory procedures, for example "[YYMMDD_lab_name_Plate#]ASGR1 Results.xlsx" in the appropriate file path then selecting 'Save'.
18. Close out the software. If prompted, save the changes to the experiment.

19. Result Interpretation by Cq review. Begin review of run data using the following chart. Positive (+) is defined as having a Cq value and Negative (-) having an NaN.

Table 14: *Table of specimen result interpretations*

SARS-CoV-2 (FAM)	Influenza A (Quasar670)	Influenza B (HEX)	RSV (Quasar705)	RNaseP (CalRed610)	Result Interpretation
-	-	-	-	-	Invalid
-	-	-	-	+	SARS-CoV-2, Influenza A, Influenza B, RSV not detected
+	-	-	-	+/-	Positive SARS-CoV-2
-	+	-	-	+/-	Positive Influenza A
-	-	+	-	+/-	Positive Influenza B
-	-	-	+	+/-	Positive RSV
+	+	-	-	+/-	Positive SARS-CoV-2 Positive Influenza A
+	-	+	-	+/-	Positive SARS-CoV-2, Positive Influenza B
+	-	-	+	+/-	Positive SARS-CoV-2, Positive RSV
-	+	+	-	+/-	Positive Influenza A, Positive Influenza B
-	+	-	+	+/-	Positive Influenza A, Positive RSV
-	-	+	+	+/-	Positive Influenza B, Positive RSV
+	+	+	-	+/-	Positive SARS-CoV-2, Positive Influenza A, Positive Influenza B
+	+	-	+	+/-	Positive SARS-CoV-2, Positive Influenza A, Positive RSV
+	-	+	+	+/-	Positive SARS-CoV-2, Positive Influenza B, Positive RSV
-	+	+	+	+/-	Positive Influenza A, Positive Influenza B, Positive RSV
+	+	+	+	+/-	Positive SARS-CoV-2, Positive Influenza A, Positive Influenza B, Positive RSV

20. Review any non-Sigmoidal or other non-standard amplification curves and rerun specimens as necessary. Baseline noise, primarily due to bubbles in wells, may present as sharp increases in fluorescence (RFU) and appear as jagged lines. If this background is present above the Cq line remove specimen(s) from the analysis and repeat test(s). Typical sigmoidal amplification curves for each target are shown in Figures 8-12.

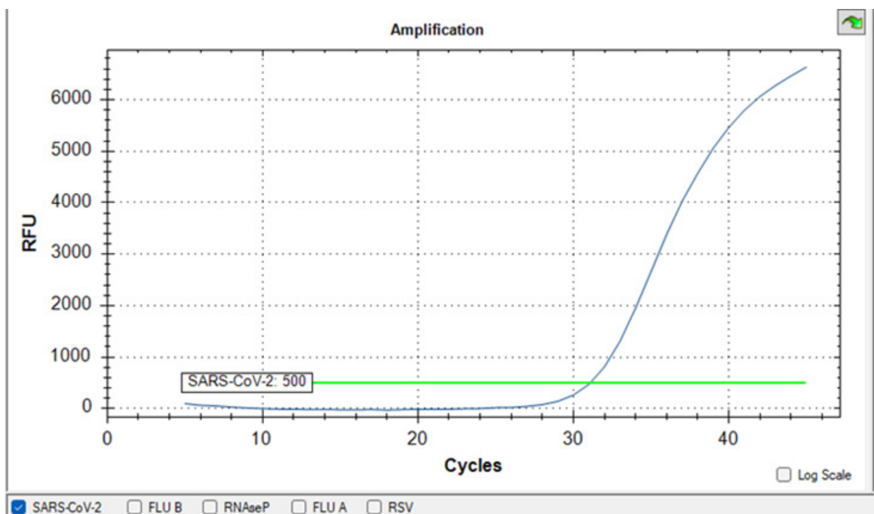


Figure 8: *Positive SARS-CoV-2 specimen amplification plot*

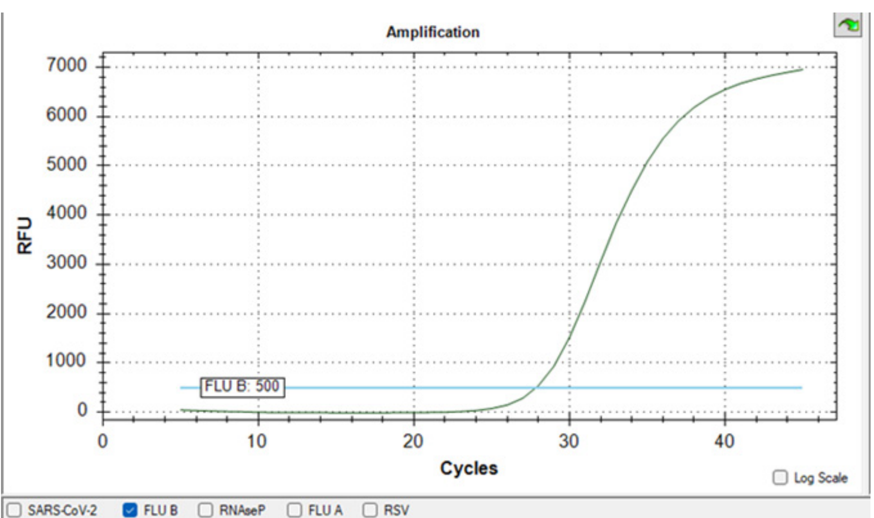


Figure 9: *Positive Influenza B specimen amplification plot*

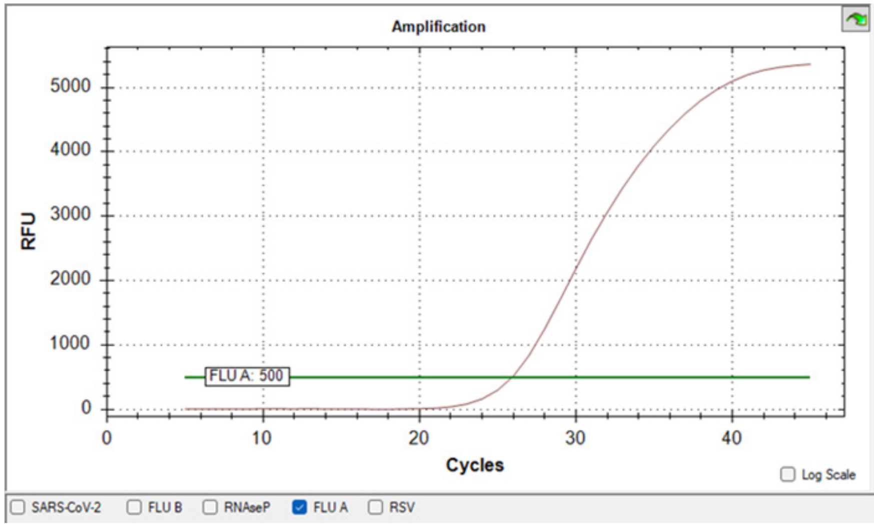


Figure 10: *Positive Influenza A specimen amplification plot*

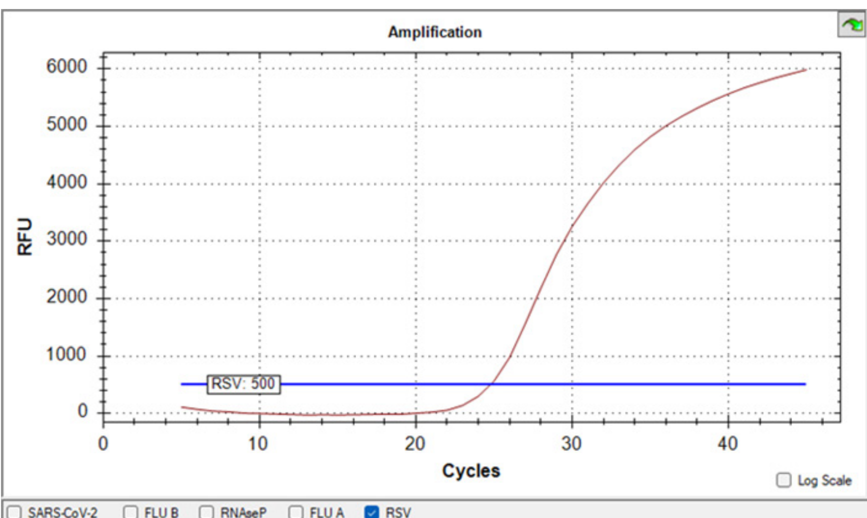


Figure 11: *Positive RSV specimen amplification plot*

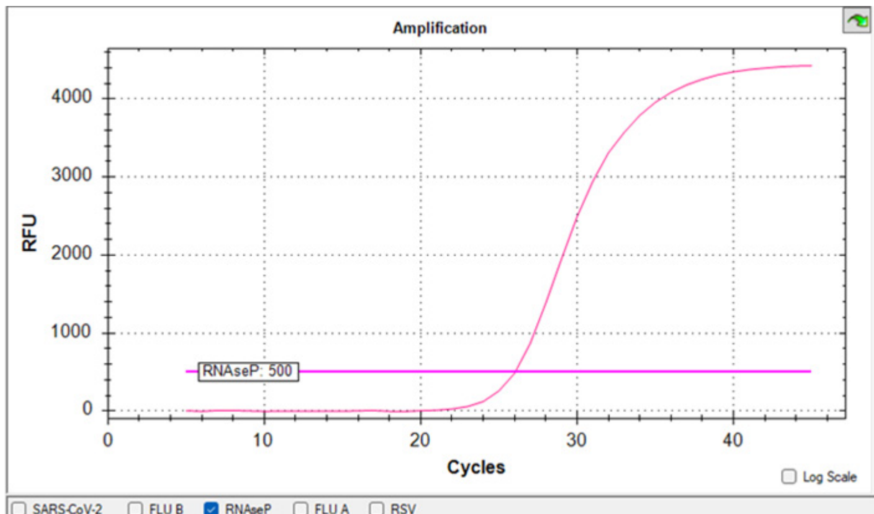


Figure 12: *Negative specimen amplification plot (Internal control RNaseP positive)*

21. After all specimens have been reviewed, select Export → Custom Export to export run data for LIMS import. If not using a LIMS system, then go to Tools → Reports and export data from there (Figure 13).
22. To create a PDF report file, Go to "Tools" in the top toolbar of the run file window and select "Reports". A window will come up with a report. On the *left side* of the window un-check "Gene Expression – Bar Chart" and then "Update Report" on the bottom left portion of the screen. After the report updates, save the PDF file.

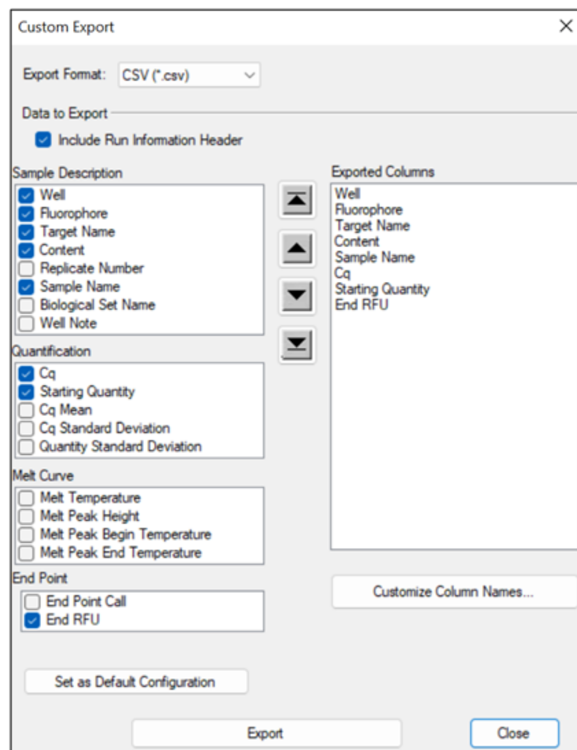


Figure 13: *Custom Export configuration*

Limitations of the Procedure

- Performance characteristics have been determined with nasopharyngeal specimens from human patients with signs and symptoms of respiratory infection only. The AmphiSense Gold Respiratory Kit 1 has not been validated for the testing of pooled specimens or the screening of specimens from asymptomatic individuals that do not have signs and symptoms of respiratory infection.
- Use of AmphiSense Gold Respiratory Kit 1 with other clinical specimen types has not been assessed and performance characteristics are unknown.
- Specimens should not be collected in saline.
- The AmphiSense Gold Respiratory Kit 1 is a qualitative test that reports Cq values for individuals that test positive for SARS-CoV-2, influenza A, influenza B, and/or RSV. These Cq values should not be interpreted as a measure of viral levels.
- Performance characteristics for influenza A were established when influenza A/H1 and A/H3 were predominant. When other influenza A viruses are emerging, performance characteristics may differ.
- This test does not differentiate influenza A subtypes (i.e., H1N1, H3N2); additional testing is required to differentiate any specific influenza A subtypes or strains, in consultation with local public health departments.
- The test is not intended to differentiate influenza B lineages. If differentiation of specific lineages is needed, additional testing, in consultation with state or local public health departments, is required.
- Due to the absence of positive results during the prospective clinical study, performance characteristics for influenza B were established with retrospective clinical specimens.
- The clinical performance has not been established with all circulating SARS-CoV-2 variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which changes over time.
- As with any molecular test, mutations within the target regions of the AmphiSense Gold Respiratory Kit 1 could affect primer and/or probe binding resulting in failure to detect the presence of virus.
- Positive and negative predictive values are highly dependent on prevalence. The likelihood of a negative result being false is higher during peak activity when prevalence of disease is high. The likelihood of a positive result being false is higher during periods when prevalence is moderate to low.
- False negative results may occur if on-panel viruses are present at levels below the analytical limit of detection.
- Negative results do not preclude SARS-CoV-2, influenza A, influenza B, or RSV infections and should not be used as the sole basis for diagnosis, treatment, or other patient management decisions.
- Detection of SARS-CoV-2, influenza A, influenza B, and RSV RNA may be affected by sample collection methods, patient factors (e.g., presence of symptoms), and/or stage of infection.
- The effect of interfering substances has only been evaluated for those listed in this labeling. Potential interference has not been evaluated for substances other than those described in the Interfering Substances section below. Interference by substances other than those described in the Interfering Substances section below could lead to erroneous results.
- Recent patient exposure to FluMist® or other live attenuated influenza vaccines may cause inaccurate positive influenza results. FluMist Vaccine, when tested alone at a concentration of 0.5% v/v in contrived nasopharyngeal specimens, was found to produce false positive results for influenza A and influenza B. FluMist vaccine when tested in at a concentration of 0.5% v/v in combination with assay targets in contrived nasopharyngeal specimens did not interfere with detection of assay targets at low levels (3x assay LoD).
- Results from analytical studies with contrived co-infected samples showed potential for competitive interference with influenza A at low concentrations (~3x LoD) when RSV concentration is >1.05e3 RNA copies/mL. In addition, there is potential for competitive interference with influenza B at low concentration (~3x LoD) and SARS-CoV-2 at low concentration (~3x LoD) when RSV concentration is >7.30e3 RNA copies/mL.

- Primers for the AmphiSense Gold Respiratory Kit 1 SARS-CoV-2 assay share 100% sequence homology in the assay amplicon region with the Bat coronavirus BANAL-20-236 (accession: MZ937003) and cross-reactivity with this closely related viral sequence is predicted. In addition, the SARS-CoV-2 assay may cross-react with another bat SARS-like coronavirus sequence (accession MG772933.1). It is unlikely that these viruses would be found in a human clinical nasopharyngeal swab; but if present, the cross-reactive product produced by the AmphiSense Gold Respiratory Kit 1 will be detected as SARS-CoV-2.
- The performance of this test was validated using the procedures provided in these Instructions for Use. Modifications to these procedures may alter the performance of the test.
- Erroneous test results might occur from improper specimen collection; failure to follow the recommended sample collection, handling, and storage procedures; technical error; or sample mix-up. Careful compliance with the instructions in this insert is necessary to avoid erroneous results.
- Good laboratory practices and careful adherence to the procedures specified in this Instructions for Use document are necessary to avoid contamination of reagents.
- Viral nucleic acid may persist in vivo, independent of virus infectivity. Detection of analyte target(s) does not imply that the corresponding virus(es) are infectious or are the causative agents for clinical symptoms.
- This test has been evaluated for use with human specimen material only.

Assay Performance Characteristics

Analytical Sensitivity (Limit of Detection)

The analytical sensitivity (limit of detection or LoD) of the AmphiSense Gold Respiratory Kit 1 was determined by testing dilutions of pooled negative clinical nasopharyngeal (NP) swab VTM/UTM matrix spiked with the following virus cultures: Influenza A (5 strains), Influenza B (2 strains), RSV A and RSV B (1 strain each), or inactivated SARS-CoV-2 (2 strains). This study was conducted with one lot of reagents. To determine the putative LoD, organisms were diluted 3x in series and a minimum of 3 replicates were tested per dilution until 3/3 replicates were detected. Confirmation of the estimated LoD was performed in replicates of 20 and the LoD was determined as the lowest detectable concentration of virus at which approximately 95% (19/20) of all replicates test positive. The highest (least sensitive) LoD value for each strain tested was reported as the final, verified LoD. The highest, (least sensitive) LoD value for each target organism was determined to be the organism LoD. The verified LoD values for the viruses tested are summarized in Table 15.

Table 15: *Summary of Limit of Detection Results*

Assay Target	Strain	LoD Concentration ($TCID_{50}$/mL)
Influenza A	Influenza A H1N1 (Brisbane/59/07)	0.041
	Influenza A H1N1 (Solomon Islands/03/06)	**3.33**
	Influenza A H3N2 Virus (Hong Kong/2671/19)	1.11
	Influenza A H3N2 (A/Kansas/14/17)	3.33
	Influenza A H1N1pdm (A/NY/02/09)	0.041
Influenza B	**Influenza B (Malaysia/2506/04)**	**0.014**
	Influenza B (Massachusetts/02/12)	0.014
RSV	**Respiratory Syncytial Virus Type A (RSV-A)**	**0.014**
	Respiratory Syncytial Virus Type B (RSV-B)	0.005
SARS-CoV-2	**SARS-CoV-2 USA-WA1/2020**	**0.123**
	SARS-CoV-2 WHO International Standard (England/02/2020)	270 (IU/mL)

Analytical Reactivity (Inclusivity)

Flu A, Flu B, RSV, and SARS-CoV-2 Inclusivity Wet-Testing

Analytical reactivity was evaluated by testing against multiple strains of influenza A H1N1, influenza A H1N1pdm (pandemic 2009), influenza A H3N2, influenza B (including strains from both Victoria and Yamagata lineages), respiratory syncytial virus subgroups A and B (RSV A and RSV B) and SARS-CoV-2, at concentrations of ~3x LoD in negative clinical NP swab matrix. A minimum of three replicates were tested for each strain. If initial testing at 3x LoD did not yield 3/3 positive replicates, concentrations were increased by threefold and strains were re-tested in replicates of three until a concentration was reached with 3/3 replicates detected. A total of 60 respiratory virus strains including 27 influenza A, 14 influenza B, 12 RSV (5 RSVA and 7 RSV B) and 7 SARS-CoV-2 strains, were evaluated for analytical reactivity. Table 16 shows the lowest concentration of each strain for which 100% positivity was observed.

Table 16: *Inclusivity Results for Target Virus Strains*

Strain	Lowest detected Concentration (units/xLoD)	Results*			
		Flu A	Flu B	RSV	SARS-CoV-2
Influenza A H1N1					
A/Solomon Islands/03/06**	3.33 $TCID_{50}$/mL (1x)	+	-	-	-
A/Brisbane/59/07**	0.041 $TCID_{50}$/mL (<1x)	+	-	-	-
A/New Caledonia/20/99	10 $TCID_{50}$/mL (3x)	+	-	-	-
Oseltamivir-R H274Y Isolate 1	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/FM/1/47	3320 cp/mL (3x)	+	-	-	-
A/Denver/1/57	3320 cp/mL (3x)	+	-	-	-
A/Mal/302/54	3320 cp/mL (3x)	+	-	-	-
A/NWS/33	3320 cp/mL (3x)	+	-	-	-
A/PR/8/34	3320 cp/mL (3x)	+	-	-	-
A/Swine/Iowa/15/30	3320 cp/mL (3x)	+	-	-	-
Influenza A H1N1pdm					
A/Swine/NY/02/2009**	0.041 $TCID_{50}$/mL (<1x)	+	-	-	-
A/Brisbane/02/2018	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Guangdong-Maonan/SWL/1536/2019	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Mexico/4108/2009	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Michigan/45/2015	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/California/07/2009	3320 cp/mL (3x)	+	-	-	-
Influenza A H3N2					
A/Kansas/14/17**	3.33 $TCID_{50}$/mL (1x)	+	-	-	-
A/Hong Kong/2617/19**	1.11 $TCID_{50}$/mL (<1x)	+	-	-	-
A/Hong Kong/4801/14	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Hong Kong/8/68	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Singapore/INFIMH-16- 0019/16	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Texas/50/2012	10 $TCID_{50}$/mL (3x)	+	-	-	-
A/Aichi/2/68	3320 cp/mL (3x)	+	-	-	-
A/Port Chalmers/1/73	3320 cp/mL (3x)	+	-	-	-
A/Victoria/3/75	3320 cp/mL (3x)	+	-	-	-
A/Wisconsin/67/2005	3320 cp/mL (3x)	+	-	-	-
Influenza A H3N2(v)					
A/Indiana/10/2011	10 $TCID_{50}$/mL (3x)	+	-	-	-
Influenza B Victoria					
B/Malaysia/2506/04**	0.014 $TCID_{50}$/mL (1x)	-	+	-	-
B/Alabama/2/17	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
B/Brisbane/46/15	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
B/Ohio/1/05	3993 cp/mL (3x)	-	+	-	-
Influenza B Yamagata					
B/Massachusetts/2/2012**	0.014 $TCID_{50}$/mL (1x)	-	+	-	-
B/Florida/04/06	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
B/Florida/07/04	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
B/Wisconsin/01/10	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
Influenza B					
B/Allen/45	0.041 $TCID_{50}$/mL (3x)	-	+	-	-
B/GL/1739/54	3993 cp/mL (3x)	-	+	-	-
B/Hong Kong/5/72	3993 cp/mL (3x)	-	+	-	-
B/Lee/40	3993 cp/mL (3x)	-	+	-	-
B/Maryland/1/59	3993 cp/mL (3x)	-	+	-	-
B/Taiwan/2/62	3993 cp/mL (3x)	-	+	-	-

Strain	Lowest detected Concentration (units/xLoD)	Results*			
		Flu A	Flu B	RSV	SARS-CoV-2
RSV A					
RSV Type A strain 2006**	0.014 $TCID_{50}$/mL (1x)	-	-	+	-
12/2014 Isolate #2	431 cp/mL	-	-	+	-
3/2015 Isolate #3	431 cp/mL	-	-	+	-
A2/Melbourne/1961	1292 cp/mL (9x)	-	-	+	-
Long/Maryland/1956	1292 cp/mL (9x)	-	-	+	-
RSV B					
RSV Type B strain/isolate from 2014**	0.005 $TCID_{50}$/mL (<1x)	-	-	+	-
18537/Washington DC/1962	431 cp/mL (3x)	-	-	+	-
3/2015 Isolate #1	1292 cp/mL (9x)	-	-	+	-
9320/Massachusetts/1977	431 cp/mL (3x)	-	-	+	-
B1	431 cp/mL (3x)	-	-	+	-
Ch-93 (18)-18	1292 cp/mL	-	-	+	-
WV/14617/1985	1292 cp/mL (9x)	-	-	+	-
SARS-CoV-2					
2019-nCoV/USA-WA1/2020**	0.123 $TCID_{50}$/mL (1x)	-	-	-	+
England/02/2020 (WHO Intl Std)**	270 IU/mL	-	-	-	+
hCoV-19/USA/MD-HP20874/2021 (Omicron)	0.37 $TCID_{50}$/mL (3x)	-	-	-	+
South_Africa/KRISP-K005325/2020 (Beta)	0.37 $TCID_{50}$/mL (3x)	-	-	-	+
USA/CA_CDC_5574/2020 (Alpha)**	0.37 $TCID_{50}$/mL (3x)	-	-	-	+
USA/NY-Wadsworth-21033899-01/2021 (Gamma)	0.37 $TCID_{50}$/mL (3x)	-	-	-	+
USA/PHC658/2021 (Delta)	0.37 $TCID_{50}$/mL (3x)	-	-	-	+

**A positive symbol (+) indicates that reactivity was observed for 100% of the replicates (3/3) while a negative symbol (-) indicates that reactivity was observed for 0% of the replicates.*
***These strains are considered to pass Inclusivity requirements based on results of testing for the LoD study. Result shown was determined during the LoD study.*

SARS-CoV-2 Inclusivity Wet-Testing – RNA Transcripts for Omicron 2022 isolates
A panel of RNA transcripts was created to evaluate the risk of mismatches to SARS-CoV-2 assay primers and probe that were identified within the Omicron variant (in July-Sept 2022).

Analytical Specificity (Exclusivity) Wet Testing

Analytical specificity (cross-reactivity) of the AmphiSense Gold Respiratory Kit 1 was evaluated in the presence of non-target viral, bacterial, or fungal organisms that may be present in a respiratory specimen. A total of 53 non-target organisms (24 viral organisms and 29 bacteria or yeast) and human nasal wash were evaluated for cross-reactivity. Organisms were diluted for testing in negative clinical NP swab matrix to the concentrations listed in Table 17. All organisms tested produced negative results for all assay target viruses when tested at the listed concentrations.

Table 17: *Cross-reactivity Results for Non-target Organisms*

Exclusivity Organism	Type	Vendor	Catalog #	Tested Concentration	Negative Results Obtained (negative result /total)
Adenovirus 1 / Adenoid 71 (Species C)	Live Virus	ZeptoMetrix	0810050CF	10^5 $TCID_{50}$/mL	3/3
Adenovirus 4 (Species E)	Live Virus	ATCC	VR-1572	10^5 $TCID_{50}$/mL	3/3
Adenovirus 7a (Species B)	Live Virus	ATCC	VR-848	10^5 $TCID_{50}$/mL	3/3
Coronavirus 229E	Live Virus	ZeptoMetrix	0810229CF	10^4 $TCID_{50}$/mL	3/3
Coronavirus HKU1	Synthetic RNA	ATCC	VR-3262SD	10^6 copies/mL	3/3
Coronavirus NL63	Live Virus	ZeptoMetrix	0810228CF	10^4 $TCID_{50}$/mL	3/3
Coronavirus OC43	Live Virus	ZeptoMetrix	0810024CF	10^4 $TCID_{50}$/mL	3/3
Cytomegalovirus (CMV / HHV 5)	Live Virus	ZeptoMetrix	0810003CF	10^4 $TCID_{50}$/mL	3/3
Enterovirus 68	Live Virus	ATCC	VR-1823PQ	10^5 $TCID_{50}$/mL	3/3
Enterovirus 71	Live Virus	ZeptoMetrix	0810236CF	10^4 $TCID_{50}$/mL	3/3
Epstein–Barr Virus (EBV)	Live Virus	ZeptoMetrix	0810008CF	10^5 $TCID_{50}$/mL	3/3
Human herpesvirus 1 (HSV-1)	Live Virus	ZeptoMetrix	0810183CF	10^4 $TCID_{50}$/mL	3/3
Human herpesvirus 2 (HSV-2)	Live Virus	ZeptoMetrix	0810006CF	10^4 $TCID_{50}$/mL	3/3
Human metapneumovirus (hMPV) A1	Live Virus	ZeptoMetrix	0810160CF	10^5 $TCID_{50}$/mL	3/3
Human metapneumovirus (hMPV) B1	Live Virus	ZeptoMetrix	0810156CF	10^3 $TCID_{50}$/mL	3/3
Human Rhinovirus type A1 (species A)	Live Virus	ZeptoMetrix	0810012CFN	10^4 $TCID_{50}$/mL	3/3
Measles virus	Live Virus	ZeptoMetrix	0810025CF	10^4 $TCID_{50}$/mL	3/3
MERS-CoV	Inactivated Virus	ATCC	NR-50549	10^4 $TCID_{50}$/mL	3/3
Mumps	Live Virus	ZeptoMetrix	0810079CF	10^5 $TCID_{50}$/mL	3/3
Parainfluenza Type 1 (HPIV 1)	Live Virus	ZeptoMetrix	0810014CF	10^5 $TCID_{50}$/mL	3/3
Parainfluenza Type 2	Live Virus	ZeptoMetrix	0810015CF	10^5 $TCID_{50}$/mL	3/3
Parainfluenza Type 3	Live Virus	ZeptoMetrix	0810016CF	10^5 $TCID_{50}$/mL	3/3
Parainfluenza Type 4a	Live Virus	ZeptoMetrix	0810060CF	10^5 $TCID_{50}$/mL	3/3
SARS-1	RNA	ATCC	vr-3280sd	10^5 $TCID_{50}$/mL	3/3
Aspergillus sp.	Live Fungus	ZeptoMetrix	801827	10^6 CFU/mL	3/3
Bordetella bronchiseptica	Live Bacteria	ZeptoMetrix	801649	10^6 CFU/mL	3/3
Bordetella parapertussis	Live Bacteria	ATCC	15311	10^6 CFU/mL	3/3
Bordetella pertussis	Live Bacteria	ZeptoMetrix	801459	10^6 CFU/mL	3/3
Candida albicans	Live Yeast	ZeptoMetrix	801504	10^6 CFU/mL	3/3
Chlamydia pneumoniae	Live Bacteria	ATCC	53592	10^6 IFU/mL	3/3
Chlamydia trachomatis	Live Bacteria	ZeptoMetrix	801775	10^6 IFU/mL	3/3
Corynebacterium diphtheriae	Live Bacteria	ZeptoMetrix	801882	10^6 CFU/mL	3/3
Escherichia coli	Live Bacteria	ZeptoMetrix	801517	10^6 CFU/mL	3/3
Fusobacterium necrophorum	Live Bacteria	ZeptoMetrix	804189	10^6 CFU/mL	3/3
Haemophilus influenzae	Live Bacteria	ATCC	33391	10^6 CFU/mL	3/3
Klebsiella pneumoniae	Live Bacteria	ATCC	BAA-2342	10^6 CFU/mL	3/3
Lactobacillus plantarum	Live Bacteria	ATCC	14917	10^6 CFU/mL	3/3
Legionella pneumophila	Live Bacteria	ZeptoMetrix	801645	10^6 CFU/mL	3/3
Moraxella catarrhalis	Live Bacteria	ATCC	25238	10^6 CFU/mL	3/3
Mycobacterium tuberculosis	Live Bacteria	ZeptoMetrix	801660	10^6 CFU/mL	3/3
Mycoplasma genitalium	Genomic DNA	ATCC	33530DQ	10^6 CFU/mL	3/3
Mycoplasma pneumoniae	Live Bacteria	ZeptoMetrix	801579	10^6 CCU/mL	3/3
Neisseria gonorrhoeae	Live Bacteria	ZeptoMetrix	801482	10^6 CFU/mL	3/3
Neisseria meningitidis	Live Bacteria	ZeptoMetrix	801511	10^6 CFU/mL	3/3

Exclusivity Organism	Type	Vendor	Catalog #	Tested Concentration	Negative Results Obtained (negative result /total)
Pneumocystis jirovecii	Live Bacteria (recombinant)	ZeptoMetrix	801698	10^6 CFU/mL	3/3
Proteus mirabilis	Live Bacteria	ATCC	35659	10^6 CFU/mL	3/3
Pseudomonas aeruginosa	Live Bacteria	ATCC	10145	10^6 CFU/mL	3/3
Staphylococcus aureus (MSSA)	Live Bacteria	ATCC	25923	10^6 CFU/mL	3/3
Staphylococcus aureus Mu50 (MRSA)	Live Bacteria	ATCC	700699D-5	10^6 CFU/mL	3/3
Staphylococcus epidermidis	Live Bacteria	ATCC	49134	10^6 CFU/mL	3/3
Streptococcus pneumoniae	Live Bacteria	ZeptoMetrix	801439	10^6 CFU/mL	3/3
Streptococcus pyogenes	Live Bacteria	ATCC	49399	10^6 CFU/mL	3/3
Streptococcus salivarius	Live Bacteria	ATCC	13419	10^6 CFU/mL	3/3
Human Nasal Wash	Nasal Wash	Lee Biosolutions	991-26-P	N/A	3/3

Analytical Specificity (Exclusivity) in silico Analysis

Primer and probe sequences for the targets of the AmphiSense Gold Respiratory Kit 1 were cross-referenced against published genome sequences for 77 non-target viruses and 59 non-target bacteria or fungi that may be present in a respiratory specimen. Primer and probe sequences for each of the targets of the AmphiSense Gold Respiratory Kit 1 were also cross-referenced against the taxid numbers for each of the other target viruses. Using BLAST, a nucleotide blast was completed with the blastn algorithm. For viruses, using the “align two or more sequences” option to individually compare each primer or probe sequence (Query Sequence) to a list of 77 cross-reactivity virus accession numbers (Subject Sequence). For bacteria and other target organisms, each primer or probe sequence (Query Sequence) was individually compared to a Search Set including Standard Databases (nucleotide collection nr/nt) and the 59 bacteria/fungi listed. No significant (potential amplicon-producing) alignments to non-target viruses or bacteria were found for influenza A, influenza B, or RSV. The SARS-CoV-2 primer and probe sequences exhibit high (>99%) homology to a Bat Coronavirus isolate (MZ937003.2) and (>90%) to other Bat coronaviruses, but no other significant alignments. The results of in silico analysis indicate that the AmphiSense Gold Respiratory Kit 1 is unlikely to cross-react with non-target viruses, bacteria, and fungi that may be present in a respiratory specimen. The Kit is, however, predicted to detect Bat Coronaviruses, although these viruses tend to be isolated to smaller geographic regions and do not actively circulate within the human population.

Microbial Interference

Organisms tested for cross-reactivity were also tested for microbial interference. A total of 53 non-target organisms (24 viral organisms and 29 bacteria or yeast) and human nasal wash were evaluated. Organisms were diluted for testing in negative clinical NP swab matrix to the concentrations listed in Table 18 in combination with pooled assay target viruses at ~3x LoD. A minimum of three replicates was tested for each organism. No microbial interference was observed for any organism.

Table 18: *Microbial Interference Results*

Organism	Test Concentration	Assay Target Results*			
		Flu A	Flu B	RSV	SARS-CoV-2
3x LoD Control	N/A	+	+	+	+
Adenovirus 1 / Adenoid 71 (Species C)	10^5 $TCID_{50}$/mL	+	+	+	+
Adenovirus 4 (Species E)	10^5 $TCID_{50}$/mL	+	+	+	+
Adenovirus 7a (Species B)	10^5 $TCID_{50}$/mL	+	+	+	+
Coronavirus 229E	10^4 $TCID_{50}$/mL	+	+	+	+
Coronavirus HKU1	10^6 copies/mL	+	+	+	+
Coronavirus NL63	10^4 $TCID_{50}$/mL	+	+	+	+
Coronavirus OC43	10^4 $TCID_{50}$/mL	+	+	+	+
Cytomegalovirus (CMV / HHV 5)	10^6 copies/mL	+	+	+	+
Enterovirus 68	10^5 $TCID_{50}$/mL	+	+	+	+
Enterovirus 71	10^4 $TCID_{50}$/mL	+	+	+	+
Epstein–Barr Virus (EBV)	10^5 copies/mL	+	+	+	+

Organism	Test Concentration	Assay Target Results*			
		Flu A	Flu B	RSV	SARS-CoV-2
Human herpesvirus 1 (HSV-1)	10^4 $TCID_{50}$/mL	+	+	+	+
Human herpesvirus 2 (HSV-2)	10^4 $TCID_{50}$/mL	+	+	+	+
Human metapneumovirus (hMPV) A1	10^5 $TCID_{50}$/mL	+	+	+	+
Human metapneumovirus (hMPV) B1	10^3 $TCID_{50}$/mL	+	+	+	+
Human Rhinovirus type A1 (species A)	10^4 $TCID_{50}$/mL	+	+	+	+
Measles virus	10^4 $TCID_{50}$/mL	+	+	+	+
MERS-CoV	10^6 copies/mL	+	+	+	+
Mumps	10^5 $TCID_{50}$/mL	+	+	+	+
Parainfluenza Type 1 (HPIV 1)	10^5 $TCID_{50}$/mL	+	+	+	+
Parainfluenza Type 2	10^5 $TCID_{50}$/mL	+	+	+	+
Parainfluenza Type 3	10^5 $TCID_{50}$/mL	+	+	+	+
Parainfluenza Type 4a	10^5 $TCID_{50}$/mL	+	+	+	+
SARS-1	10^5 copies/mL	+	+	+	+
Aspergillus sp.	10^6 CFU/mL	+	+	+	+
Bordetella bronchiseptica	10^6 CFU/mL	+	+	+	+
Bordetella parapertussis	10^6 CFU/mL	+	+	+	+
Bordetella pertussis	10^6 CFU/mL	+	+	+	+
Candida albicans	10^6 CFU/mL	+	+	+	+
Chlamydia pneumoniae	10^6 CFU/mL	+	+	+	+
Chlamydia trachomatis	10^6 CFU/mL	+	+	+	+
Corynebacterium diphtheriae	10^6 CFU/mL	+	+	+	+
Escherichia coli	10^6 CFU/mL	+	+	+	+
Fusobacterium necrophorum	10^6 CFU/mL	+	+	+	+
Haemophilus influenzae	10^6 CFU/mL	+	+	+	+
Klebsiella pneumoniae	10^6 CFU/mL	+	+	+	+
Lactobacillus plantarum	10^6 CFU/mL	+	+	+	+
Legionella pneumophila	10^6 CFU/mL	+	+	+	+
Moraxella catarrhalis	10^6 CFU/mL	+	+	+	+
Mycobacterium tuberculosis	10^6 CFU/mL	+	+	+	+
Mycoplasma genitalium	10^6 copies/mL	+	+	+	+
Mycoplasma pneumoniae	10^6 CCU/mL	+	+	+	+
Neisseria gonorrhoeae	10^6 CFU/mL	+	+	+	+
Neisseria meningitidis	10^6 CFU/mL	+	+	+	+
Pneumocystis jirovecii	10^6 CFU/mL	+	+	+	+
Proteus mirabilis	10^6 CFU/mL	+	+	+	+
Pseudomonas aeruginosa	10^6 CFU/mL	+	+	+	+
Staphylococcus aureus (MSSA)	10^6 CFU/mL	+	+	+	+
Staphylococcus aureus Mu50 (MRSA)	10^6 CFU/mL	+	+	+	+
Staphylococcus epidermidis	10^6 CFU/mL	+	+	+	+
Streptococcus pneumoniae	10^6 CFU/mL	+	+	+	+
Streptococcus pyogenes	10^6 CFU/mL	+	+	+	+
Streptococcus salivarius	10^6 CFU/mL	+	+	+	+
Human Nasal Wash	N/A	+	+	+	+

**A positive symbol (+) indicates that positivity was observed for 100% of the replicates while a negative symbol (-) indicates that positivity was observed for 0% of the replicates.*

Competitive Inhibition

Competitive inhibition in the AmphiSense Gold Respiratory Kit 1 was evaluated using pairs of the target viruses at low/high concentrations in pooled negative nasopharyngeal swab matrix. The low concentration evaluated was at 3x LoD and the high concentration evaluated was at 1E4 TCID50/mL (Influenza A, Influenza B, RSV) or 1E5 copies/mL (SARS-CoV-2). Competitive inhibition was observed with low concentrations (~3x LoD) of Influenza A and high concentrations of RSV (>1.05e3 RNA copies/ mL). In addition, there is potential for competitive interference with influenza B at low concentration (~3x LoD) and SARS-CoV-2 at low concentration (~3x LoD) when RSV concentration is >7.30e3 RNA copies/mL (this competition was evidenced by Cq shift rather than missed replicates). Results are presented in Table 19.

Table 19: *Summary of Competitive Inhibition*

Assay Target at 3x LoD	Highest Concentration of each Target for which 3/3 3x LoD target reps were detected			
	Influenza A	**Influenza B**	**RSV**	**SARS-CoV-2**
Influenza A	NA	Flu A detected with 9.57E+08 cp/mL (1E+04 TCID50/mL) Flu B	Flu A detected with 1.05E+03 cp/mL (1E-1 TCID50/mL) RSV	Flu A detected with 1E+05 cp/mL (2.33E+01 TCID50/mL) SARS-CoV-2
Influenza B	Flu B detected with 3.32E+06 cp/mL (1E+04 TCID50/mL) Flu A	NA	Flu B detected with 1.05E+08 cp/mL (1E+04 TCID50/mL) RSV	Flu B detected with 1E+05 copies/mL (2.33E+01 TCID50/mL) SARS-CoV-2
RSV	RSV detected with 3.32E+06 cp/mL (1E+04 TCID50/mL) Flu A	RSV detected with 9.57E+08 cp/mL (1E+04 TCID50/mL) Flu B	NA	RSV detected with 1E+05 copies/mL (2.33E+01 TCID50/mL) SARS-CoV-2
SARS-CoV-2	SARS-CoV-2 detected with 3.32E+06 cp/mL (1E+04 TCID50/mL) Flu A	SARS-CoV-2 detected with 9.57E+08 cp/mL (1E+04 TCID50/mL) Flu B	SARS-CoV-2 detected with 1.05E+08 cp/mL (1E+04 TCID50/mL) RSV	NA

Interfering Substances (Interference)

Endogenous and exogenous substances that may be present in a clinical nasopharyngeal swab specimen were evaluated for potential interference with the AmphiSense Gold Respiratory Kit 1 in the absence and presence of targets (Influenza A, Influenza B, RSV, and SARS-CoV-2) at 3x of their respective LoD concentrations in pooled negative nasopharyngeal swab matrix. Flu-Mist Vaccine which contains live attenuated Influenza virus was found to interfere at the tested concentration, as expected. There was no reportable interference from all other substances evaluated.

Table 20: *Endogenous and Exogenous Substances Tested with the AmphiSense Gold Respiratory Kit 1*

Substance Type	Substance	Concentration Tested	Positive Testing (Positive/Total)				Negative Testing (Negative/ Total)	Result
			Influenza A	Influenza B	RSV	SARS-CoV-2		
Endogenous Substances	Human Genomic DNA	20 ng/µL	3/3	3/3	3/3	3/3	3/3	No Interference
	Human Whole Blood	10% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
	Mucin	60 µg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	PBMCs (Leukocytes)	1000 cells/µL	3/3	3/3	3/3	3/3	3/3	No Interference
Influenza Vaccine Live, Intranasal	Flu-Mist	0.5% v/v	3/3	3/3	3/3	3/3	0/3	Interference
Nasal Corticosteroids	Beclomethasone	15 ng/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Budesonide	5% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
	Dexamethasone	12 µg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Flunisolide	10 µg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Fluticasone Propionate	5% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
	Mometasone	0.5 ng/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Triamcinolone	5% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
Nasal Sprays	Neo-synephrine (Phenylephrine)	1% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
	Oxymetazoline HCl	1% v/v	6/6	6/6	6/6	6/6	3/3	No Interference
	Saline nasal spray	1% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
Homeopathic Nasal Gel	Zicam Allergy (active ingredients Luffa Operculata, Galphimia Glauca, Histaminum Hydrochloricum, and Sulphur)	5% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
Antibiotic, Nasal Ointment	Mupirocin	2% v/v	3/3	3/3	3/3	3/3	3/3	No Interference
Antibacterial, systemic	Tobramycin	0.6 mg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
Anti-viral drugs	Ribavirin	20 mg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Tamiflu (Oseltamivir)	7.5 mg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Zanamivir	3.3 mg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
Throat lozenges / oral anesthetic and analgesic	Benzocaine	3 mg/mL	6/6	6/6	6/6	6/6	3/3	No Interference
	Menthol Throat lozenge	1.7 mg/mL	3/3	3/3	3/3	3/3	3/3	No Interference
	Zinc lozenge	5% w/v	3/3	3/3	3/3	3/3	3/3	No Interference
Analgesic NSAID	Ibuprofen	21.9 mg/dL	3/3	3/3	3/3	3/3	3/3	No Interference
Tobacco Product	Snuff	1% w/v	3/3	3/3	3/3	3/3	3/3	No Interference
Swab	Swab-Floq tip	N/A	3/3	3/3	3/3	3/3	3/3	No Interference
	Swab-Foam tip	N/A	3/3	3/3	3/3	3/3	3/3	No Interference
	Swab-Rayon tip	N/A	3/3	3/3	3/3	3/3	3/3	No Interference

Carry-over Contamination

A carry-over contamination study was conducted with each individual target at high concentrations (1.0×10^4 $TCID_{50}$/mL for FluA, FluB, and RSV or 1.0×10^5 copies/mL for SARS-CoV-2) in pooled negative nasopharyngeal swab matrix. The high concentration samples were extracted and plated in a checkerboard pattern that alternated with negative samples (nasopharyngeal swab matrix). A minimum of 5, 96-well plates were run for each target over a period of 5 non-consecutive days. Each plate contained 47 positive samples and 46 negative samples, plus 3 controls. There was no detectable carry-over contamination when samples were extracted using the KingFisher™ platform with MagMAX™ Viral/Pathogen II Nucleic Acid Isolation extraction reagents and PCR conducted on the BioRad CFX 96 Dx.

Single-site Precision

Within laboratory precision was evaluated for the AmphiSense Gold Respiratory Kit 1 at one site with one reagent lot. The precision was established using a 9-member panel prepared in simulated negative nasopharyngeal matrix which included 3 negative samples, 3 low positive (~2x LoD) and 3 moderate positive (~5x LoD) samples. The positive samples contained all 4 targets combined (Influenza A, Influenza B, RSV, and SARS-CoV-2). The panels were blinded and evaluated by 2 operators each performing 2 runs per day over a total of 12 non-consecutive days. Results for the Precision evaluation are presented in Tables 21 (quantitative) and 22 (qualitative).

Table 21: *Overall Quantitative Precision Results*

Target	Panel Member	N	Mean	Within Run		Between Days		Between Operators		Between Runs/ Instruments		Total	
				SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%
FluA	2x LoD	92	36.67	1.31	3.57	1.31	3.58	1.31	3.57	1.48	4.02	1.48	4.03
	5x LoD	95	34.97	1.35	3.87	1.44	4.13	1.41	4.04	1.35	3.87	1.50	4.29
FluB	2x LoD	96	33.93	0.64	1.89	0.64	1.90	0.66	1.95	0.70	2.07	0.72	2.13
	5x LoD	96	32.39	1.24	3.83	1.33	4.10	1.24	3.83	1.24	3.83	1.33	4.10
RSV	2x LoD	96	35.52	1.60	4.50	1.71	4.81	1.60	4.50	1.84	5.18	1.94	5.45
	5x LoD	96	34.06	1.50	4.41	1.53	4.50	1.55	4.56	1.50	4.41	1.58	4.65
SARS-CoV-2	2x LoD	96	36.26	0.76	2.10	0.77	2.12	0.79	2.17	0.81	2.24	0.84	2.32
	5x LoD	96	34.94	0.63	1.80	0.63	1.80	0.71	2.05	0.63	1.81	0.72	2.06

Table 22: *Overall Qualitative Precision Results*

Assay Target	Panel member	Expected Result	Overall Precision Result		
			% Positive	% agreement with expected	95% CI
FluA	**Moderate Positive (5x LoD)**	**100% Positive**	98.96% (95/96)	98.96*	94.33-99.82
	Low Positive (2x LoD)	**≥95% Positive**	95.83% (92/96)	95.83**	89.77-98.37
	Negative	**Negative**	0% (0/96)	100	96.15-100
FluB	**Moderate Positive (5x LoD)**	**100% Positive**	100% (96/96)	100	96.15-100
	Low Positive (2x LoD)	**≥95% Positive**	100% (96/96)	100	96.15-100
	Negative	**Negative**	0% (0/96)	100	96.15-100
RSV	**Moderate Positive (5x LoD)**	**100% Positive**	100% (96/96)	100	96.15-100
	Low Positive (2x LoD)	**≥95% Positive**	100% (96/96)	100	96.15-100
	Negative	**Negative**	0% (0/96)	100	96.15-100
SARS-CoV-2	**Low Positive (2x LoD)**	**100% Positive**	100% (96/96)	100	96.15-100
	Moderate Positive (5x LoD)	**≥95% Positive**	100% (96/96)	100	96.15-100
	Negative	**Negative**	0% (0/96)	100	96.15-100

**98.96% agreement, 1 Flu A NEG out of 96 samples, no trend observed for operator, day, or instrument study components. The 98.96% point estimate falls within the 95% Wilson CI for a sample size of 96 (96.15 – 100).*
***95.83%, 4 Flu A NEG, equal distribution b/t operator, instrument day. 95.83% positivity rate meets the ≥95% expected based on the concentration for 1-2x LoD samples.*

Reproducibility – Reagent Lot-to-Lot

The reproducibility and repeatability of the AmphiSense Gold Respiratory Kit 1 was evaluated across four reagent kit lots. Lot-to-lot reproducibility was established using a 9-member panel prepared in simulated negative nasopharyngeal matrix which included 3 negative samples, 3 low positive (~2x LoD) and 3 moderate positive (~5x LoD) samples. The positive samples contained all 4 targets combined (Influenza A, Influenza B, RSV, and SARS-CoV-2). The panels were evaluated by 1 operator performing 2 runs per day over a total of 3 non-consecutive days. Results for the Lot-to-lot reproducibility evaluation are presented in Tables 23 (Variability Analysis) and 24 (Overall Percent Agreement).

Table 23: *Lot to Lot Reproducibility Study Variability Analysis Results*

Target	Panel Member	N	Mean	Within Run (residual error)		Between Lot		Between Day		Between Run		Total	
				SD	CV%	SD	CV%	SD	CV%	SD	CV%	SD	CV%
FluA	2x LoD	70	37.07	0.83	2.24	0.83	2.24	1.04	2.82	1.30	3.51	1.45	3.91
	5x LoD	72	34.49	1.07	3.09	1.07	3.09	1.13	3.27	1.43	4.15	1.47	4.28
FluB	2x LoD	72	34.10	0.31	0.90	0.31	0.90	0.44	1.29	0.43	1.27	0.54	1.57
	5x LoD	72	32.61	0.44	1.36	0.44	1.36	0.45	1.37	0.61	1.88	0.61	1.88
RSV	2x LoD	72	35.88	1.18	3.29	1.18	3.29	1.18	3.29	1.57	4.39	1.57	4.39
	5x LoD	72	34.44	0.49	1.42	0.49	1.42	0.85	2.48	0.65	1.88	0.95	2.77
SARS-CoV-2	2x LoD	72	36.23	0.62	1.70	0.63	1.75	0.62	1.70	0.68	1.88	0.70	1.93
	5x LoD	72	34.53	0.46	1.33	0.46	1.33	0.55	1.59	0.49	1.42	0.58	1.67

Table 24: *Overall Lot-to-Lot Reproducibility Study Results Using Four Lots of AmphiSense Gold Respiratory Kit 1 (Percent Agreement with Expected Results)*

Target	Panel Member	Expected Result	Kit Lot 005	Kit Lot 006	Kit Lot 006b	Kit Lot 007	Overall
Influenza A	5x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	2x LoD	Positive	88.89% (16/18)*	100% (18/18)	100% (18/18)	100% (18/18)	97.22% (70/72) 90.43-99.23
	Negative	Negative	0% (0/18)	0% (0/18)	0% (0/18)	0% (0/18)	100% (0/72) 94.93-100
Influenza B	5x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	2x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	Negative	Negative	0% (0/18)	0% (0/18)	0% (0/18)	0% (0/18)	100% (0/72) 94.93-100
RSV	5x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	2x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	Negative	Negative	0% (0/18)	0% (0/18)	0% (0/18)	0% (0/18)	100% (0/72) 94.93-100
SARS-CoV-2	5x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	2x LoD	Positive	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)	100% (72/72) 94.93-100
	Negative	Negative	0% (0/18)	0% (0/18)	0% (0/18)	0% (0/18)	100% (0/72) 94.93-100

**With sample size of 18, Wilson 95% CI for Expected result 18/18 POS = 82.41%-100%. 2x LoD Lot 005 result of 88.89% falls within the 95% CI for this sample size*

Reproducibility

The reproducibility and repeatability of the AmphiSense Gold Respiratory Kit 1 was evaluated across three external sites using one lot of reagents. Reproducibility was established using a 9-member panel prepared in simulated negative nasopharyngeal matrix which included 3 negative samples, 3 low positive (~2x LoD) and 3 moderate positive (~5x LoD) samples. The positive samples contained all 4 targets combined (Influenza A, Influenza B, RSV, and SARS-CoV-2). The panels were blinded and evaluated by 2 operators per site with each operator performing 2 runs per day over a total of 5 non-consecutive days. Results for the Reproducibility evaluation are presented in Tables 25 (Overall Quantitative Results) and 26 (Overall Qualitative Results).

Table 25: *Overall Quantitative Reproducibility Results*

Assay Target	Panel Member	Mean Cq	N	Within Run		Between Site		Between Operator/ Run		Between Day		Reproducibility	
				SD	CV %	SD	CV %	SD	CV %	SD	CV %	SD	CV %
Flu A	2x LoD	35.87	90	1.19	3.33	1.37	3.83	1.22	3.41	1.20	3.35	1.41	3.92
	5x LoD	34.12	90	1.02	2.99	1.26	3.69	1.09	3.21	1.02	2.99	1.32	3.87
Flu B	2x LoD	34.19	90	1.00	2.92	1.21	3.55	1.00	2.92	1.01	2.97	1.22	3.58
	5x LoD	32.97	90	0.56	1.69	0.73	2.22	0.56	1.69	0.65	1.98	0.81	2.45
RSV	2x LoD	36.20	86	1.31	3.61	1.37	3.79	1.31	3.61	1.52	4.19	1.57	4.35
	5x LoD	34.69	90	1.07	3.09	1.07	3.09	1.11	3.20	1.21	3.50	1.25	3.59
SARS-CoV-2	2x LoD	36.52	90	0.97	2.67	1.00	2.74	0.97	2.67	1.14	3.11	1.16	3.18
	5x LoD	34.97	90	0.48	1.38	0.48	1.38	0.48	1.38	0.74	2.13	0.74	2.13

Table 26: *Overall Qualitative Reproducibility Results*

Assay Target	Panel Member	Expected Result	Site 1		Site 2		Site 3		Overall	
			% Agreement with Expected Result		% Agreement with Expected Result		% Agreement with Expected Result		% Agreement with Expected Result	
Influenza A	2x LoD	≥95% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	5x LoD	100% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	Negative	Negative	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
Influenza B	2x LoD	≥95% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	5x LoD	100% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	Negative	Negative	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
RSV	2x LoD	≥95% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	86.67% (26/30)	(70.32-94.69%)	95.56% (86/90)	(89.12-98.26%)
	5x LoD	100% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	Negative	Negative	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
SARS-CoV-2	2x LoD	≥95% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	5x LoD	100% Positive	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)
	Negative	Negative	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100 % (30/30)	(88.65-100%)	100% (90/90)	(95.91-100%)

Clinical Performance

The clinical performance of the AmphiSense Gold Respiratory Kit 1 was established in a multi-center study conducted with residual (leftover) and de-identified NP swab specimens that were prospectively collected from patients with signs and symptoms of respiratory tract infections during periods of the 2022-2023 respiratory seasons. NP swab specimens from four geographically diverse clinical sites in the U.S. were enrolled and tested with the AmphiSense Gold Respiratory Kit 1 at four U.S. testing sites. A total of 3,200 specimens were enrolled throughout the duration of this study and occurred across two different respiratory seasons. The frozen prospective specimen enrollment began at two collection-only sites and one of the four testing sites on March 15, 2022, and ended on May 20, 2022. A second frozen prospective enrollment began at two collection-only sites on December 14, 2022, and ended January 25, 2023. This resulted in a total enrollment of 1,337 frozen prospective specimens. From March 15, 2022 to March 6, 2023, 1,863 fresh prospective specimens were enrolled. In addition, thirty (30) positive Influenza B specimens that were collected between 2017 and 2018, and thirty (30) negative specimens that were collected on May 20, 2022 were enrolled as retrospective specimens to supplement the data at the end of the study. Retrospective specimens were randomized and tested at two (2) clinical testing sites in January and March 2023. A total of 240 specimens were excluded for Influenza A/B and RSV and a total of 239 specimens for SARS-CoV-2 due to screen failures during enrollment or other reasons including shipping delays, sample collection in incompatible media, and unable to obtain a comparator result. The final data set consisted of 2960 prospective specimens and 60 retrospective specimens. Table 27 summarizes the age demographic information for the 2960 specimens included in the prospective study. Table 28 summarizes the gender distribution for all of the enrolled specimens, minus screen failures (total of 3191 specimens).

Table 27: *Demographic Age Summary by Target*

Analyte		Positive Percent Agreement			Negative Percent Agreement		
		TP/(TP+FN)	%	95% CI	TN/(TN+FP)	%	95%CI
Flu A	Birth to 5 years	21/22	95.45	78.20-99.20	198/199	99.50	97.21-99.91
	6-21 years	45/46	97.83	88.67-99.62	215/215	100.00	98.25-100.00
	22-59 years	90/93	96.77	90.94-98.90	1214/1216	99.84	99.40-99.95
	60 years and older	37/40	92.50	80.14-97.42	1128/1129	99.91	99.50-99.98
Flu B	Birth to 5 years	0/0	N/A	N/A	221/221	100.00	98.29-100.00
	6-21 years	0/0	N/A	N/A	261/261	100.00	98.55-100.00
	22-59 years	0/0	N/A	N/A	1309/1309	100.00	99.71-100.00
	60 years and older	0/0	N/A	N/A	1169/1169	100.00	99.67-100.00
RSV	Birth to 5 years	19/22	86.36	66.67-95.25	199/199	100.00	98.11-100.00
	6-21 years	5/6	83.33	43.65-97.00	255/255	100.00	98.52-100.00
	22-59 years	22/24	91.67	74.15-97.69	1285/1285	100.00	99.70-100.00
	60 years and older	19/21	90.48	71.09-97.35	1148/1148	100.00	99.67-100.00
SARS-CoV-2	Birth to 5 years	11/11	100.00	74.12-100.00	210/210	100.00	98.50-100.00
	6-21 years	18/26	69.23	50.01-83.50	235/235	100.00	98.39-100.00
	22-59 years	207/232	89.22	84.58-92.59	1285/1295	99.23	98.58-99.58
	60 years and older	185/204	90.69	85.91-93.96	956/965	99.07	98.24-99.51

Table 28: *Gender Demographic Summary*

Gender	Total specimens	% Total specimens
Male	1340	41.99%
Female	1850	57.98%
Unidentified	1	0.03%

Specimens were tested using the AmphiSense Gold Respiratory Kit 1 and were compared side-by-side to a U.S. FDA-cleared molecular respiratory panel to determine the Positive Percent Agreement (PPA) and Negative Percent Agreement (NPA). The overall PPA and NPA for the AmphiSense Gold Respiratory Kit 1 for Influenza A is 96.02% and 99.86%, respectively; N/A and 100% for Influenza B, respectively; 89.04% and 100% for RSV, respectively; 89.01% and 99.24% for SARS-CoV-2, respectively (Table 29).

Table 29: *Fresh and Frozen Prospective Performance – All Sites*

Analyte		Positive Percent Agreement			Negative Percent Agreement		
		TP/(TP+FN)	%	95% CI	TN/(TN+FP)	%	95%CI
Flu A	Fresh Prospective	115/119	96.64	91.68-98.69	1622/1623	99.94	99.65-99.99
	Frozen Prospective	78/82	95.12	88.12-98.09	1133/1136	99.74	99.23-99.91
	Overall	193/201	96.02	92.34-97.97	2755/2759	99.86	99.63-99.94
Flu B	Fresh Prospective	0/0	N/A	N/A	1742/1742	100.00	99.78-100.00
	Frozen Prospective	0/0	N/A	N/A	1218/1218	100.00	99.69-100.00
	Overall	0/0	N/A	N/A	2960/2960	100.00	99.87-100.00
RSV	Fresh Prospective	50/57	87.72	76.75-93.92	1685/1685	100.00	99.77-100.00
	Frozen Prospective	15/16	93.75	71.67-98.89	1202/1202	100.00	99.68-100.00
	Overall	65/73	89.04	79.84-94.34	2887/2887	100.00	99.87-100.00
SARS-CoV-2	Fresh Prospective	204/220	92.73	88.51-95.47	1513/1522	99.41	98.88-99.69
	Frozen Prospective	217/253	85.77	80.93-89.54	956/966	98.96	98.11-99.44
	Overall	*421/473	89.01	85.87-91.52	*2469/2488	99.24	98.81-99.51

**Performance at one site for SARS-CoV-2 testing was an outlier, as the overall performance for this site was much lower when compared to the other three testing sites. 30/40 SARS-CoV-2 false negatives at this site were concordant with the SOC result, in comparison to the remaining SARS-CoV-2 false negative SARS-CoV-2 specimens from all other sites where 6/12 were concordant with the SOC result. This site accounted for 76.9% (40/52) of the false negatives included in the analysis.*

7/13 SARS-CoV-2 false positives at this site were concordant with the SOC result, in comparison to the remaining SARS-CoV-2 false positives at all other sites where 2/6 were concordant with the SOC result. This site accounted for 36.8% (7/19) of the false positives included in the analysis. Without this site's testing data included in the final dataset, the following performance would have been observed:

Influenza A PPA	168/174 = 96.55%	(95% CI = 92.68% to 98.41%)
Influenza A NPA	1790/1794 = 99.78%	(95% CI = 99.43% to 99.91%)
RSV PPA	62/69 = 89.86%	(95% CI = 80.51% to 95.00%)
RSV NPA	1899/1899 = 100.00%	(95% CI = 99.80% to 100.00%)
SARS-CoV-2 PPA	281/293 = 95.90%	(95% CI = 92.98% to 97.64%)
SARS-CoV-2 NPA	1669/1675 = 99.64%	(95% CI = 99.22% to 99.84%)

Frozen retrospective specimens, 30 positives and 30 negatives, were evaluated to support the clinical data set for Influenza B. The specimens were randomized and tested at two different sites. The PPA and NPA for Influenza B on the AmphiSense Gold Respiratory Kit 1 was 100.00% (Table 30).

Table 30: *Frozen Retrospective Specimen Performance*

Analyte	PPA			NPA		
	TP/(TP+FN)	%	95% CI	TN/(TN+FP)	%	95%CI
Flu B	30/30	100.00	88.65-100.00	30/30	100.00	88.65-100.00

The number of specimens with positive results for more than one target as detected by AmphiSense Gold Respiratory Kit 1 and the comparator test is presented in Table 31. The percent of coinfections detected during testing is presented in Table 32.

Table 31: *Coinfections Detected in Prospectively Collected Specimens*

Coinfection Combination	# of Specimens Detected by AmphiSense Gold Respiratory Kit 1	# of Specimens Detected by Comparator
Influenza A + SARS-CoV-2	3	6
Influenza A + RSV	1	1
Total	**4**	**7**

Table 32: *Percent Coinfections Detected During Prospective Collection and Testing*

	# Positive Specimens	# Total Specimens
# Coinfections	7/740	7/2960
% Coinfections	0.95%	0.24%

References

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6. Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19). CDC. 13 Dec 2021.

Revision History

Revision	Date	Change Summary
01	11/30/2023	Initial release

Trademarks

AmphiSense™ is a trademark of SeqOnce Biosciences.
All other product names and trademarks are the property of their respective owners.

Explanation of Symbols

The following symbols are present on the AmphiSense Gold Respiratory Kit 1 labels and kits.

Symbol	Definition
5.1.1	Manufacturer
5.3.7	Temperature limit
5.1.5 LOT	Batch code
QTY	Quantity
5.1.6 REF	Catalog Number
5.1.4	Use-by Date
5.4.3	Consult instructions for use
5.4.1	Biological risks
5.5.1 IVD	In vitro diagnostic medical device

Manufacturing and Distribution Information

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